PATENT COOPERATIOI\ ?EATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 25 August 2000 (25.08.00)	
International application No. PCT/US99/19068	**Applicant's or agent's file reference** 6523-020-228
International filing date (day/month/year) 18 August 1999 (18.08.99)	**Priority date** (day/month/year) 18 August 1998 (18.08.98)
Applicant XU, Tian et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   16 March 2000 (16.03.00)

   [ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was
   [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer Alejandro HENNING Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: ADRIANE M. ANTLER
PENNIE & EDMONDS LLP
1155 AVENUE OF THE AMERICAS
NEW YORK, NY 10036
USA

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing *(day/month/year)*	31 JAN 2001
Applicant's or agent's file reference: 6523-020-228	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/US99/19068	18 AUGUST 1999	18 AUGUST 1998

Applicant

YALE UNIVERSITY

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	SHIN-LIN CHEN *[signature] for*
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/416 (July 1992)★

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 6523-020-228	**FOR FURTHER ACTION** See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US99/19068	International filing date *(day/month/year)* 18 AUGUST 1999	Priority date *(day/month/year)* 18 AUGUST 1998
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant YALE UNIVERSITY		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 8 sheets.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description. claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☒ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
16 MARCH 2000	28 DECEMBER 2000
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SHIN-LIN CHEN Della Collins for Telephone No. (703) 308-0196

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/19068

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-54, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of ______

[X] the claims:
pages 55-67, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of ______

[X] the drawings:
pages 1-51, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of ______

[X] the sequence listing part of the description:
pages 1-37, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of ______

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language ______ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[X] contained in the international application in printed form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application.

☒ claims Nos. 24-67, 82, 83, 93, 99-113

because:

☐ the said international application, or the said claim Nos. _ relate to the following subject matter which does not require international preliminary examination *(specify)*.

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. _ are so unclear that no meaningful opinion could be formed *(specify)*.

☐ the claims, or said claims Nos. _ are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for said claims Nos. (See Attached) .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/19068

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

- [ ] restricted the claims.
- [X] paid additional fees.
- [ ] paid additional fees under protest.
- [ ] neither restricted nor paid additional fees.

2. [ ] This Authority found that the requirement of unity of invention is not complied with and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

- [ ] complied with.
- [X] not complied with for the following reasons:

Please See Supplemental Sheet.

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

- [ ] all parts.
- [ ] the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	(Please See supplemental sheet)	YES
	Claims	(Please See supplemental sheet)	NO
Inventive Step (IS)	Claims	(Please See supplemental sheet)	YES
	Claims	(Please See supplemental sheet)	NO
Industrial Applicability (IA)	Claims	(Please See supplemental sheet)	YES
	Claims	(Please See supplemental sheet)	NO

2. citations and explanations (Rule 70.7)

Claims 1, 3, 5, 6, 8, 10-16, 18, 20 and 22 lack an inventive step under PCT Article 33(3) as being obvious over Yale University, 1996 (A) in view of Ligand Pharmaceuticals, Inc. (B), 1995.

Reference A teaches that lats gene encodes a protein which acts as a tumor suppressor to inhibit cell proliferation. Reference A teaches generation of a non-human animal comprising an inactivated lats gene via homologous recombination of nucleic acid comprising a non-lats sequence flanked by lats genomic sequence (e.g. p. 162, 163), and use of said non-human knockout animal as an animal model for studying diseases and disorders involving cell overproliferation (e.g. malignancy) and screening for test molecule having the ability to inhibit overproliferation (e.g. tumor formation) and to treat or prevent such diseases or disorders (e.g. p. 78, 79). Reference A also teaches a method of treating or preventing a disease or disorder involving cell proliferatiion, such as breast cancer, colon cancer, bladder cancer sarcoma, melanoma etc., in a subject by administering a therapeutically effective amount of a molecule that promote lats function (e.g. p. 158).

Reference A does not specifically teaches comparing the size of a cancer in a recombinant non-human animal with or without the administration of the potential therapeutic compound.

Reference B teaches a method for screening for a therapeutic agent for treatment of a pathological condition affected by the level of a cytokine by measuring the binding of a protein to a promoter, wherein a reduction in the binding in the presence of said agent compared to the binding in the absence of said agent is indicative of said agent as potentially useful for treatment of said condition.

It would have been obvious for one of ordinary skill at the time of the invention to use the non-human knockout animal as an animal model for screening agent as taught by A and compare the size of the cancer or tumor in said knockout animal in the presence or absence of said agent in order to identify a therapeutic compound that could promote lats function such as to provide therapeutic effects for the treatment of diseases or disorders involving cell overproliferation. such as cancers.

(Continued on Supplemental Sheet.)

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

The description is objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 5 because it fails to adequately enable practice of the claimed invention because: the description only discloses a knockout mouse lacking functional lats gene (lats-/-) and fails to provide adequate suidance and evidence for any other non-human knockout animal lacking functional lats gene. The art in producing transgenic animal was unpredictable at the time the invention was made. One skilled in the art would not be able to predict the resulting phenotype of a non-human knockout lats-/- animal, and would require undue experimentation to practice over the full scope of the invention claimed.

Further, the description fails to provide adequate guidance and evidence for the therapeutic effects of a complex or a chimeric protein of a lats protein and a cdc2 protein. The art for gene therapy or protein therapy was unpredictable at the time of the invention. It would have required one skilled in the art undue experimentation to practice the claimed invention.

Claims 1-7, 10-17, 20-23, 89-92 and 94-98 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because practice of the claimed invention is not enabled as required under PCT Rule 5.1(a) for the reasons set forth in the immediately preceding paragraph.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/19068

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII | Sheet 10

CLASSIFICATION:
The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): A61K 39/395; C07H 21/04; A01N 61/00, 37/18, 43/04; C12N 5/00, 15/00 and US Cl.: 424/130.1; 536/23.5; 514/1, 2, 44; 800/3, 13, 14, 21, 25

III. NON-ESTABLISHMENT OF REPORT:

No international search report has been established for claim numbers 24-67, 82, 83, 93, 99-113.

IV. LACK OF UNITY OF INVENTION:

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2, and 13.3 is not complied with for the following reasons:
This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-23, drawn to recombinant non-human animal with knockout lats gene function, method of using said animal for screening compound in treating cancer.

Group II, claim(s) 68-77 and 89-91, drawn to a purified complex of a lats protein and cdc2 protein, and a pharmaceutical composition containing said complex.

Group III, claim(s)78-81, 86, 88, 92, 95 and 97, drawn to a chimeric protein comprising fragment of lats protein and cdc2 protein, and a pharmaceutical composition containing said chimeric protein.

Group IV, claim(s) 84, 85, 87, 94, 96 and 98, drawn to a combination of nucleic acids comprising a nucleic acid encoding a lats protein and a nucleic acid encoding a cdc2 protein, and a pharmaceutical composition containing said nucleic acid.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
Transgenic animals, nucleic acids and proteins are different products which differ in physical properties, chemical structure and utilities, and do not share any common special technical feature. Groups I, II-III and IV do not share special technical features.
A chimeric protein comprising fragment of a lats protein and a cdc2 protein differs from a complex of protein comprising a lats protein and a cdc2 protein structurally and chemically. They are different products with different functions and different usages. Groups II and III do not share special technical features. Thus, Groups I-IV do not relate to a single inventive concept.

V. 1. REASONED STATEMENTS:
The report as to Novelty was positive (YES) with respect to claims 1-23, 68-81, 84-92, 94-98.
The report as to Novelty was negative (NO) with respect to claims NONE.
The report as to Inventive Step was positive (YES) with respect to claims 2, 4, 7, 9, 17, 19, 21, 23, 68-81, 84-92, 94-98.
The report as to Inventive Step was negative (NO) with respect to claims 1, 3, 5, 6, 8, 10-16, 18, 20, 22.
The report as to Industrial Applicability was positive (YES) with respect to claims 1-23, 68-81, 84-92, 94-98.
The report as to Industrial Applicability was negative (NO) with respect to claims NONE.

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
Claims 1-23, 68-81, 84-92 and 94-98 meet the criteria set out in PCT Article 33(2) and (4), because the prior art does not

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 11

teach or fairly suggest the combination of lats protein or nucleic acid with cdc2 protein or nucleic acid and the combination of lats and cdc2.

------------------- NEW CITATIONS -------------------
NONE

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: ADRIANE M. ANTLER
PENNIE & EDMONDS LLP
1155 AVENUE OF THE AMERICAS
NEW YORK, NY 10036
USA

REFERRED TO
REC'D
DEC 2 0 1999
Pennie & Edmonds
O.K. for filing

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

	Date of Mailing *(day/month/year)* 16 DEC 1999
Applicant's or agent's file reference 6523-020-228	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/US99/19068	International filing date *(day/month/year)* 18 AUGUST 1999
Applicant YALE UNIVERSITY	

1. [X] The applicant is hereby notified that the international search report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 *bis* 1 and 90 *bis* 3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	SHIN-LIN CHEN Della Collins for
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/220 (January 1994)★ *(See notes on accompanying sheet)*

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 6523-020-228	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US99/19068	International filing date *(day/month/year)* 18 AUGUST 1999	(Earliest) Priority Date *(day/month/year)* 18 AUGUST 1998
Applicant YALE UNIVERSITY		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (See Box I).

2. [X] **Unity of invention is lacking** (See Box II).

3. [X] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [X] filed with the international application.
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] transcribed by this Authority.

4. With regard to the **title**,
   - [ ] the text is approved as submitted by the applicant.
   - [X] the text has been established by this Authority to read as follows:

LATS KNOCK-OUT ANIMAL MODELS AND THEIR USES

5. With regard to the **abstract**,
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. ______
   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19068

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19068

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :424/130.1; 536/23.5; 514/1, 2, 44; 800/3, 13, 14, 21, 25
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/130.1; 536/23.5; 514/1, 2, 44; 800/3, 13, 14, 21, 25

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, STN, MEDLINE, CAPLUS, BIOSIS, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 96/30402 A1 (YALE UNIVERSITY) 03 October 1996, entilre document, especially pages 158-163.	1-5 6-113
Y	WO 95/31722 A1 (LIGAND PHARMACEUTICALS, INC.) 23 November 1995, entire document, especially page 50.	6-37
Y, P	TAO et al. Human homologue of the Drosophila melanogaster lats tumor suppressor modulates CDC2 activity, Nature Genetics, February 1999, Vol 21, No. 2, page 177-181, entire document.	68-113

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 OCTOBER 1999	16 DEC 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SHIN-LIN CHEN Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	ST. JOHN et al. Mice Deficient of Lats1 Develop Soft-Tissue Sarcomas, Ovarian Tumors and Pituitary Dysfunction, Nature Genetics, February 1999, Vol 21, No. 2, page 182-186, entire document.	1-37

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19068

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 39/395; C07H 21/04; A01N 61/00, 37/18, 43/04; C12N 5/00, 15/00

PCT

ᴙLD INTELLECTUAL PROPERTY ORGANIZA
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 39/395, C07H 21/04, A01N 61/00, 37/18, 43/04, C12N 5/00, 15/00

A1

(11) International Publication Number: WO 00/10602

(43) International Publication Date: 2 March 2000 (02.03.00)

(21) International Application Number: PCT/US99/19068

(22) International Filing Date: 18 August 1999 (18.08.99)

(30) Priority Data:

60/096,996	18 August 1998 (18.08.98)	US
60/096,997	18 August 1998 (18.08.98)	US

(71) Applicant *(for all designated States except US)*: YALE UNIVERSITY [US/US]; 451 College Street, New Haven, CT 06520 (US).

(72) Inventors; and

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(54) Title: LATS KNOCK-OUT ANIMAL MODELS AND THEIR USES

(57) Abstract

A recombinant non-human animal having an inactivated *lats* gene is described. A *lats* knock-out mouse is exemplified. Because mice disrupted for the *lats* gene develop a variety of tumors, are susceptible to induction of skin tumors by exposure to carcinogens, and exhibit pituitary dysfunction, they have utility in screening for compounds effective to treat or prevent cancer or pituitary disorders. Compounds can be screened for activity in treating or preventing skin cancer in recombinant non-human animals which have an inactivated and in which skin tumors have been induced by exposure to carcinogens. Methods for treatment of cancers refractory to treatment with chemotherapy or radiation therapy by using a therapeutic that promotes lats function are also described. Additional methods are described for the treatment or prevention of diseases and disorders associated with aberrant levels of cdc2 activity with a therapeutic that either promotes, inhibits or antagonizes lats function.

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LATS KNOCK-OUT ANIMAL MODELS AND THEIR USES

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RELATED APPLICATIONS

Priority is claimed to United States provisional application Serial Nos. 60/096,997 and 60/096,996, both filed on August 18, 1998, both of which are incorporated by reference herein in their entireties.

FIELD OF THE INVENTION

The present invention relates to the use of lats proteins, derivatives and fragments for the treatment of cancer, particularly for the treatment of cancer that is refractory to treatment by standard chemotherapy and radiation therapy protocols. The present invention also relates to the use of lats proteins, derivatives and fragments for the treatment of diseases and disorders associated with an aberrantly high or aberrantly low level of cdc2 activity. The present invention further provides complexes of lats and cdc2, and their production and uses. The present invention also provides an animal model for cancer, particularly for skin cancer, soft tissue sarcomas, and ovarian tumors, and for pituitary disorders. The animal model is preferably a mouse, in which a *lats* gene has been disrupted by homologous recombination, *e.g.*, a *lats* knock-out mouse. The present invention also provides methods of screening potential therapeutics for efficacy in the treatment and prevention of cancer and pituitary disorders using *lats* knock-out animals.

BACKGROUND OF THE INVENTION

Cancer

A neoplasm, or tumor, is a neoplastic mass resulting from abnormal uncontrolled cell growth, which may cause swelling on the body surface, and which can be benign or malignant. Benign tumors generally remain localized. Malignant tumors are collectively termed cancers. The term "malignant" generally means that the tumor can invade and destroy neighboring body structures and spread to distant sites to cause death (for review, see Robbins and Angell, 1976, *Basic Pathology*, 2d Ed., W.B. Saunders Co., Philadelphia, pp. 68-122). Treatment options, such as surgery, chemotherapy and radiation treatment, are

either ineffective or present serious side effects. Thus, there is a need for development of new drugs for the treatment of cancer.

The Cell Cycle and Tumor Suppressors

Many cancers have been linked to perturbations in the regulation of the cell cycle, resulting in deregulation of cell growth. Briefly, the cell cycle occurs in four stages: G1 (for Gap1), the resting stage prior to DNA synthesis; S (for synthesis) phase, in which DNA synthesis occurs; G2 (for Gap2), the resting stage after DNA synthesis and prior to mitosis; and M phase, mitosis, in which cell division occurs. Progression of the cell cycle is driven by a group of cyclin-dependent kinases (CDKs) (Elledge, 1996, Science 274:1664-1672; Nasmyth, 1996, Science 274:1643-1645). The kinase activities of CDKs require their positive subunits, the cyclins, and the activities of specific CDK/cyclin complexes are in turn positively and negatively regulated by phosphorylation events and CDK inhibitors (CKIs) (Hunter and Pines, 1995, Cell 80:225-236; Morgan, 1995, Nature 374:131-134). While the specific CDKs, CDK2, CDK4 and CDK6, along with Cyclins D and E, regulate the progression from G1 into S phase, cdc2, along with Cyclins A and B, regulate the cell cycle progression from G1 into mitosis (Hunter and Pines, 1995, Cell 80:225-236).

Human tumor suppressors often act as negative regulators of the cell cycle, and several tumor suppressors are known to affect the activities of the CDK/cyclin complexes. For example, p53 activates the transcription of the p21 ($p21^{WAF1/CIP1}$) CDK inhibitor in response to DNA damage signals, and p21 in turn binds and inactivates the CDK4 and CDK6 cyclin D complexes (Gartel et al., 1996, Proc. Soc. Exp. Biol. Med. 213:138-149). Another CDK inhibitor, p16, is itself a potent tumor suppressor (Biggs and Kraft, 1995, J. Mol. Med. 73:509-514). Although multiple members of the p16 and p21 inhibitor families have been identified for other major CDKs, corresponding inhibitors that regulate the mitotic CDK, cdc2, have not previously been identified (Morgan, 1995, Nature 374:131-134).

Cancer Therapy

Currently, cancer therapy may involve surgery, chemotherapy and/or radiation treatment to eradicate neoplastic cells in a patient (see, for example, Stockdale, 1998, "Principles of Cancer Patient Management", in Scientific American: Medicine, vol. 3, Rubenstein and Federman, eds., Chapter 12, Section IV). All of these approaches pose significant drawbacks for the patient. Surgery, for example, may be contraindicated due to the health of the patient or may be unacceptable to the patient. Additionally, surgery may not completely remove the neoplastic tissue. Radiation therapy is only effective when the neoplastic tissue exhibits a higher sensitivity to radiation than normal tissue, and radiation therapy can also often elicit serious side effects.

With respect to chemotherapy, there are a variety of chemotherapeutic agents available for treatment of neoplastic disease. A significant majority of cancer chemotherapeutics act by inhibiting DNA synthesis, either directly, or indirectly by inhibiting the biosynthesis of the deoxyribonucleotide triphosphate precursors, to prevent DNA replication and concomitant cell division (see, for example, Gilman et al., Goodman and Gilman's: The Pharmacological Basis of Therapeutics, Eighth Ed. (Pergamom Press, New York, 1990)). These agents, which include alkylating agents, such as nitrosourea, anti-metabolites, such as methotrexate and hydroxyurea, and other agents, such as etoposides, campathecins, bleomycin, doxorubicin, daunorubicin, etc., although not necessarily cell cycle specific, kill cells during S phase because of their effect on DNA replication. Other agents, specifically colchicine and the vinca alkaloids, such as vinblastine and vincristine, interfere with microtubule assembly resulting in mitotic arrest. Chemotherapy protocols generally involve administration of a combination of chemotherapeutic agents to increase the efficacy of treatment.

Despite the availability of a variety of chemotherapeutic agents, chemotherapy has many drawbacks (see, for example, Stockdale, 1998, "Principles Of Cancer Patient Management" in Scientific American Medicine, vol. 3, Rubenstein and Federman, eds., ch. 12, sect. 10). Almost all chemotherapeutic agents are toxic, and chemotherapy causes significant, and often dangerous, side effects, including severe nausea, bone marrow depression, immunosuppression, etc. Additionally, even with administration of combinations of chemotherapeutic agents, many tumor cells are resistant or develop resistance to the chemotherapeutic agents. In fact, those cells resistant to the particular chemotherapeutic agents used in the treatment protocol often prove to be resistant to other drugs, even those agents that act by mechanisms different from the mechanisms of action of the drugs used in the specific treatment; this phenomenon is termed pleiotropic drug or multidrug resistance. Thus, because of drug resistance, many cancers prove refractory to standard chemotherapeutic treatment protocols. There is a significant need for alternative cancer treatments, particularly for treatment of cancer that has proved refractory to standard cancer treatments, such as surgery, radiation therapy, and chemotherapy.

Pituitary Disorders

The pituitary regulates numerous biological functions through its secretion of different hormones. (For review see Frohman, "The Anterior Pituitary" in Cecil Textbook of Medicine, 18th Ed., Wyngaarden and Smith, eds. (W.B. Saunders Company, Philadelphia, 1988) pp. 1290-1305). In particular, the pituitary releases glycoprotein hormones, which include luteinizing hormone (LH) and follicle stimulating hormone (FSH); LH and FSH regulate ovarian and testicular development as well as reproductive functions such as ovulation and spermatogenesis. Disruption of LH or FSH secretion has dramatic consequences for reproductive function, particularly for ovulation in the female. The pituitary also releases somatomammotropic hormones, including growth hormone and

prolactin. Growth hormone promotes linear growth and is involved in the regulation of certain metabolic functions such as sugar and amino acid uptake and use of fat stores. Prolactin stimulates and maintains lactation in post-parturition females. Although an increase or decrease in prolactin levels does not appear to have significant biological consequences beyond an effect on lactation, disruption of growth hormone secretion stunts growth and has other metabolic effects. Other pituitary hormones include corticotropin (ACTH), thyroid stimulating hormone (TSH), and endorphins and related peptides. Although in some situations, hormone replacement therapy is available, there is a need for additional therapeutics to treat or prevent pituitary dysfunctions.

LATS

The *large tumor suppressor* or *lats* gene (also known as *warts*), a tumor suppressor gene, was previously isolated from *Drosophila* using a mosaic screen. Inactivation of *lats* in somatic cells causes dramatic overproliferation phenotypes (Xu et al., 1995, Development 121:1053-1063; Justice et al., 1995, Genes & Devel. 9:534-546). Somatic cells that are mutant for *lats* undergo extensive proliferation and form large tumors in many tissues of mosaic flies (Xu et al., 1995, Development 121:1053-1063). Tumors that result from inactivation of *lats* display many features of human neoplasms. *Lats* mutant cells grow aggressively, and a single mutant cell can develop into a tumor that is 1/5 the size of the animal, and these fly tumors are highly irregular in shape and size and are often poorly differentiated (St. John and Xu, 1997, Am. J. Hum. Genet 61:1006-1010). *Drosophila* that are homozygous for the various *lats* alleles display a wide range of developmental defects including embryonic lethality, overproliferation of both neural and epidermal tissues, rough eyes, and sterility. Molecular characterization of lats indicates that it contains a putative kinase domain (Xu et al., 1995, Development 121:1053-1063; Justice et al., 1995, Genes & Devel. 9:534-546).

Mouse and human homologs of the *Drosophila lats* have also been identified, and human *lats* was found to be down-regulated in a large number of human tumor cell lines. The nucleotide and amino acid sequences of human lats (h-lats), mouse lats (m-lats), mouse lats2 (m-lats2) and *Drosophila* lats are provided herein in Figures 12-15, respectively (SEQ ID NOS:1-8, respectively), and are described in PCT Publication WO 96/30402, published October 3, 1996, which is incorporated by reference herein in its entirety.

Citation of references hereinabove shall not be construed as an admission that such references are prior art to the present invention.

SUMMARY OF THE INVENTION

The present invention relates to therapeutic and prophylactic methods and compositions for the treatment and prevention of cancers based on lats proteins, and therapeutically and/or prophylactically effective analogs and fragments of lats protein. This is due to the fact that although most tumor suppressor genes regulate the G1/S phase of the

cell cycle, the lats protein interacts with the cell cycle-dependent kinase cdc2, which is involved in the regulation of the G2 to M transition of the cell cycle, and thereby provides a means to regulate the G2 to M transition of the cell cycle and to treat cancers that have proven refractory to other cancer treatments, including chemotherapy and radiation therapy treatments. The invention provides for treatment and prevention of cancer by administration of a therapeutic compound of the invention. The therapeutic compounds of the invention useful for treatment of cancer refractory to a chemotherapy and/or radiation therapy protocol include: lats proteins, and therapeutically effective analogs and derivatives (including fragments) of lats, nucleic acids encoding lats proteins and therapeutically effective analogs and derivatives of lats, and lats agonists.

The invention further provides assays, both *in vivo* and *in vitro*, for testing the efficacy of the therapeutics of the invention for treatment of cancer, particularly cancer that has been shown to be refractory to chemotherapy and radiation therapy treatments.

In another aspect, the invention provides compositions and methods of production of complexes of lats and cdc2 proteins ("lats-cdc2 complexes"), including complexes of lats analogs or derivatives and cdc2 analogs and derivatives (including complexes of lats proteins with cdc2 analogs and derivatives and vice versa), where the analogs and derivatives have the ability to interact with the other member of the complex.

The phosphorylated form of lats complexes with cdc2. Accordingly, in a preferred embodiment, the lats-cdc2 complexes contain phosphorylated lats protein, specifically lats protein phosphorylated on a serine or threonine residue within 20 residues upstream of an Ala-Pro-Glu consensus in subdomain eight of a lats kinase domain, *e.g.*, corresponding to serine 909 of human lats, as depicted in Figure 12 (SEQ ID NO:2). Alternatively, the lats protein in the lats-cdc2 complex has a glutamate or aspartate residue substituted for a serine or threonine residue within 20 residues upstream of an Ala-Pro-Glu consensus in subdomain eight of a lats kinase domain, *e.g.*, corresponding to serine 909 of human lats, as depicted in Figure 12 (SEQ ID NO:2).

In another embodiment, the lats-cdc2 complex contains a portion of lats protein corresponding to amino acids 15-585 of human lats, as depicted in Figure 12 (SEQ ID NO:2).

The invention further provides methods of modulating the activity of cdc2 using lats proteins, as well as lats derivatives and fragments able to interact with cdc2 protein, lats-cdc2 complexes, and antibodies against lats-cdc2 complexes. In particular, the invention provides methods for treating or preventing disorders involving an aberrant level of cdc2 in a subject. Therapeutically effective amounts of compounds are administered to promote or inhibit LATS function, as required.

The invention provides recombinant non-human animals in which a *lats* gene has been inactivated, preferably recombinant mice in which a *lats* gene (preferably a gene having lats coding sequence of SEQ ID NO:3) has been inactivated, i.e., a *lats* knock-out mouse.

In a preferred embodiment, the invention provides a *lats* knock-out mouse in which the inactivated *lats* gene had the coding sequence of SEQ ID NO:3, prior to disruption, and in a more preferred embodiment, the inactivated *lats* gene is deleted for the Lats C-terminal domain 1 (LCD1), the Lats C-terminal domain 2 (LCD2), the Lats C-terminal domain 3 (LCD3), and all or a portion of the kinase domain, and retains the Lats flanking domain (LFD), the Lats split domain 1 (LSD1), the Lats split domain 2 (LSD2), and the putative SH3-binding domain, in a most preferred embodiment the *lats* gene is disrupted by replacement of a non-lats sequence for the sequence encoding the amino acids corresponding to amino acids 756 to 1130 of human lats, as depicted in Figure 12 (SEQ ID NO:2). In other embodiments, the inactivated *lats* gene is deleted for all or a portion of the kinase domain (*e.g.*, so as to inactivate kinase activity). A lats "knock-out" animal is an animal in which at least one genomic copy of a *lats* gene has been inactivated by insertional mutagenesis, *e.g.*, by homologous recombination, for example, as described and exemplified herein.

The invention further provides methods for screening potential therapeutics for activity in the treatment or prevention of cancer, preferably soft tissue sarcomas and ovarian tumors, using the *lats* knock-out animals of the invention. The invention also provides methods for screening potential therapeutics for activity in the treatment or prevention of pituitary dysfunctions, using the *lats* knock-out animals of the invention. In a preferred embodiment, the invention provides methods for screening potential therapeutics for activity in the treatment or prevention of skin cancer using a non-human *lats* knock-out animal, preferably a *lats* knock-out mouse, in which skin tumors have been induced with carcinogens.

Definitions

As used herein, underscoring or italicizing the name of a gene shall indicate the gene, in contrast to its encoded protein product which is indicated by the name of the gene in the absence of any underscoring or italicizing. For example, "*lats*" shall mean the *lats* gene, whereas "lats" shall indicate the protein product of the *lats* gene.

ARN = After Removal of Nocodazole

CDK = Cyclin Dependent Kinase

mlats = mouse lats

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-H. Human *lats* can functionally replace the fly gene. (A) Adult *Drosophila* in which *lats* homozygous mutant cells have been induced in the imaginal tissues of the *lats* heterozygous larvae, exhibit *lats* mosaic phenotype, and have mutant cells which have undergone extensive proliferation and formed tumors in various body parts of the mosaic adults. (B) Adult *Drosophila* that express human lats (hs-h-lats) completely do not exhibit tumor development (compare to A). (C, D) High magnification views of the

flies in panel (B) show *yellow* bristles (white arrows), indicating lats cells (genetically marked by the *yellow* mutation) were produced and that they have developed into normal structures. (E, F) *lats*$^{e26\text{-}1}$ homozygous mutants display giant pupae and disc-overproliferation phenotypes (left pupae in panels). Without heat-shock induction, the leaky expression of the *hs-h-lats* transgene partially suppressed the *lats*$^{e26\text{-}1}$ phenotypes (middle pupae in panels). Daily induction of the *hs-h-lats* gene completely rescued the mutant phenotypes (right pupae in panels). (G) Scanning Election Micrograph view of a *lats* mosaic fly. (H) A *lats* tumor on the wing (indicated by an arrow in panel G) is enlarged, showing that cells in the overproliferated mutant clone have differentiated into wing cells with hair structures.

Figures 2A-E. Phosphorylation of lats oscillates with the cell cycle. (A) The phosphorylation of lats protein in HeLa cells after exposure of the cells to certain conditions was assayed by immunoprecipitation and blotted with an anti-h-lats monoclonal antibody. "CIP" indicates that the cells were incubated in calf intestinal phosphatase and "ß-gp" indicates that the cells were incubated in ß-glycerol phosphate. The "Time (min.)" indicates time in minutes of incubation. "Ⓟ-h-Lats" and "h-Lats" indicate the phosphorylated and dephosphorylated forms of h-lats, respectively. Lats proteins from mitotic HeLa cell lysates (50 min. ARN (After Removal of Nocodazole)) display a slow-migrating form on SDS-PAGE (6%) (lane 1). The proteins are converted into a fast-migrating form when incubated with Calf Intestinal Phosphatase ("CIP") (lanes 2-4). When both CIP and a CIP inhibitor, ß-glycerol phosphate ("ß-gp"), are present, their mobility remains unchanged (lane 5). Lats proteins from 125 min ARN cells have both the slow-migrating and fast-migrating forms (lane 6) and CIP-treatment converts all lats proteins into the fast-migrating form (lanes 7-9). (B) Immunowestern blot shows that phosphorylation of the lats protein oscillates with the cell cycle. Cell cycle stages G0, G1, S, and G2, are indicated above each lane and cells in different mitotic stages (M) are indicated by min. (minutes ARN). The faint bands are degradation products of lats. The progression of the cell cycle was verified by DAPI staining. (C-E) These panels show fluorescent micrographs of DAPI staining for cells at three time points (50' (C), 75' (D), and 100' (E) ARN). Arrows indicate cells at metaphase (50' ARN (panel C)), anaphase (75' ARN (panel D)), or telophase (100' ARN (panel E)), respectively.

Figures 3A-E. Lats directly complexes with cdc2 during mitosis and the lats/cdc2 complex is inactive for H1 kinase activity. (A) Cdc2 is co-immunoprecipitated with lats from mitotic CHO cell lysates (M) but not from quiescent CHO cell lysates (G0). Anti-h-lats polyclonal antibodies or anti-human cyclin B monoclonal antibodies were used for immunoprecipitation, and anti-human cdc2 monoclonal antibodies were used to visualize cdc2. (B) Cdc2 co-immunoprecipitated with human lats proteins at early mitosis. The stages of the cell cycle are indicated above each lane as "min. ARN" or "G0". Lats proteins were immunoprecipitated using anti-h-lats monoclonal antibodies and were separated on 8% SDS-PAGE. The western blot was sequentially probed with anti-human cdc2

monoclonal antibodies (lower panel, labeled "Cdc2"), anti-h-lats monoclonal antibodies (upper panel, labeled "h-lats"), anti-human cyclin B and cyclin A monoclonal antibodies (middle panels, labeled "Cyclin B" and "Cyclin A", respectively). (C) Co-immunoprecipitation of baculovirus-expressed human lats and cdc2 proteins. H-lats proteins were precipitated with anti-h-lats monoclonal antibodies and probed with anti-human cdc2 monoclonal antibodies (upper panel, labeled "Cdc 2") and cdc2 proteins were precipitated with anti-human cdc2 monoclonal antibodies and probed with anti-h-lats monoclonal antibodies (lower panel, labeled "h-lats"). (D) Lats-associated cdc2 is inactive for H1 kinase activity. Cdc2 proteins co-immunoprecipitated with either lats or cyclin B (indicated by "+" in the legend labeled "anti-Cyclin B" or "anti-h-lats") from 50 min. ARN HeLa cell lysates were divided for western quantification of cdc2 (upper panel, labeled "IP-Western") and for the histone H1 kinase assay (lower panel, labeled "H1 Kinase assay"), respectively. As a control, Protein G-agarose beads incubated with equal amounts of cell lysates only were also used for the H1 kinase assay (indicated by "-" in the legend). (E) Summarized are the results of yeast two-hybrid assays for interactions among full-length h-lats (h-lats), N-terminal region of h-lats (N-h-lats), C-terminal region of h-lats (C-h-lats), human cdc2, CDK2, and CDK4. +++, ++, +: indicate strong, intermediate, and weak interactions, respectively, while - and ND indicate no interaction and not determined, respectively.

Figures 4A-F. Genetic interaction between *lats*, *cdc2*, and *cyclin A* in *Drosophila*. (A) $lats^{P8}/lats^{P8}$ homozygotes die at the pupal stage. (B) Removal of one copy of the *cdc2* gene rescues $lats^{P8}$ lethality ($lats^{P8}/lats^{P8}$; $+/cdc2^{B47}$). (C) A typical rough, overproliferated eye dissected from a $lats^{P8}/lats^{P8}$ dead pupa. (D) An eye from the fly in panel (B), showing that the eye phenotype has been almost completely suppressed. (E) $lats^{e26\text{-}1}/lats^{e26\text{-}1}$ pupae (the pupa labeled 1) are much larger than wild-type (the pupa labeled 4). The giant-pupa phenotype is partially reduced by a *cdc2* temperature sensitive mutant at room temperature (pupa labeled 2) ($lats^{e26\text{-}1}/lats^{e26\text{-}1}$; $cdc2^{E1\text{-}24}/cdc2^{E1\text{-}24}$) or by removal of one copy of the *cdc2* gene ([i[a labeled 3) ($lats^{e26\text{-}1}/lats^{e26\text{-}1}$; $+/cdc2^{B47}$). (F) The third instar larval wing discs dissected from animals of the same genotypes in (E) and labeled with the same number. The lats disc overproliferation phenotype (larva 1) is dramatically suppressed by mutations in the *cdc2* gene (larvae 2 and 3). Removal of one copy of the *cyclin A* gene resulted in a phenotypic suppression of the *lats* mutants in a manner similar to removal of one *cdc2* gene as shown above.

Figures 5A-N. Effect of inactivation and overexpression of lats on the cell cycle in *Drosophila*. (A) *Drosophila* third instar eye imaginal disc contains a homozygous $lats^{x1}$ clone (arrowhead indicates the lack of Myc staining) that crosses the morphogenetic furrow (MF) (arrow). (B) Cyclin A staining (indicated by arrowhead) in the clone exhibits expression that spans the MF (arrow). (C) Composite staining of the same disc shown in panels A and B showing Myc stains and propidium iodide staining which more clearly delineates the MF and the *lats* mutant clone (indicated by the arrow) that spans it. (D)

Composite staining magnification of the same *lats* mutant clone shown in panels A-C. Cyclin A staining clearly spans the MF region (arrow). (E) Composite staining of the same region shown in panel D viewed with Myc and propidium iodide. (F) Composite staining magnification of the same clone shown in panel E viewed with Cyclin A staining and propidium iodide. Cyclin A is degraded in *lats* mutant cells at late mitosis (as indicated by the arrowheads). (G) *Drosophila* third instar eye disc containing homozygous $lats^{x1}$ clones (lack of Myc staining is indicated by arrowheads) that span the MF region. (H) The third instar eye disc shown in panel G was also stained for Cyclin B. The MF (indicated by the arrow) is well defined and free of cyclin B staining. (I and J) Scanning electron micrograph of a wild-type *Drosophila* adult eye (I) in comparison to an eye (J) of a *GMR-d-lats* transgenic fly showing fewer and irregular ommatidia and missing bristles. (K) A section of a *GMR-d-lats* eye reveals that many pigment cells are missing, and that ommatidia occasionally lack a full complement of photoreceptor cells (arrow). (L) Propidium iodide staining of a *GMR-d-lats* third instar eye imaginal disc. A stripe of intensely stained nuclei which are tetraploid (indicated by the star) are seen in the region of the second mitotic wave, which is immediately followed posteriorly by apoptotic cells with fragmented nuclei (indicated by the small arrows). (M) BrdU labeling (green) of a *GMR-d-lats* third instar eye disc reveals that S phase occurs in the second mitotic wave (indicated by the star) posterior to the MF (indicated by the arrow) just as it would in wild type. (N) A *GMR-p21* eye disc is shown in which cell proliferation in the second mitotic wave is blocked before S phase and the stripe of BrdU labeling posterior to the MF (indicated by the arrow) is abolished.

Figures 6A-D. Targeting of the *lats* locus by homologous recombination. (A) Sequence alignment of human *lats* (h-*lats*) and mouse *lats* (m-*lats*, partial sequence). Arrow indicates the point at which the mouse *lats* gene was disrupted. (B) Targeting vector for positive-negative selection of homologous recombinants at the lats locus, with restriction map and the structure of the targeted lats locus. The vector is represented by the second line from the top, while the wild-type and mutant (i.e., disrupted) lats alleles are indicated by the top and bottom lines, respectively. The BamHI sites are indicated by "B", the EcoRI sites are indicated by "R", and the EcoRV sites are indicated by "RV". Exons are represented by filled rectangles. A BamHI/EcoRV double digest generates a 3.5 kb fragment from the wild-type allele and a 5.8 kb fragment from the disrupted allele, both of which are recognized by the probe shown, which is not contained in the targeting vector. In the vector and the mutant allele, the PGK-TK gene cassette and the PGK-neo fragment are denoted by open boxes labeled accordingly. (C) Southern blot of genomic DNA isolated from individual embryonic stem cell clones. The genotypes of the clones are indicated above the lanes with the "+/+" indicating wild-type clones, "+/-" indicating clones heterozygous for the mutant allele, and "-/-" indicating clones homozygous for the mutant allele. (D) Western blot using anti-h-lats polyclonal antibody on lysates from 13.5 dpc (days post coitus) mouse embryonic fibroblasts indicating the absence of lats protein in the knock-out mice. The genotype of the clones is indicated above the lanes as in panel C.

Figures 7A and B. Growth retardation of *lats*$^{-/-}$ mice. (A) Representative picture of a *lats*$^{-/-}$ mouse (agouti, the mouse on the right) with its wild-type littermate (black, the mouse on the left) (12-days-old). (B) Representative growth curve of *lats*$^{+/+}$, *lats*$^{+/-}$, and *lats*$^{-/-}$ mice. Mice were weighed (grams (g)) at intervals and plotted against age in days.

Figures 8A-D. Ovarian phenotypes of *lats*$^{-/-}$ mice. Histopathological sections of ovaries derived from *lats*$^{+/+}$ (panels A and C) and *lats*$^{-/-}$ (panels B and D) females. Overview images are in panels A and B while high magnification views are in panels C and D. The paraffin sections were stained with hematoxylin and eosin. An absence of corpora lutea (CL) is evident in the *lats*$^{-/-}$ ovary. Ovarian stromal cell tumors (SC) which obliterate the normal structure of the ovary, eliminating follicles (FC) progressively, are readily apparent.

Figures 9A-F. Absence of mammary gland development in *lats*$^{-/-}$ Mice. (A,B) *Lats*$^{+/+}$ female with normal mammary gland and nipple development. (D,E) *Lats*$^{-/-}$ female displaying absence of mammary gland and nipple formation. (C,F) Hematoxylin and eosin stained histopathological sections of mammary glands derived from *lats*$^{+/+}$ (C) and *lats*$^{-/-}$ (F) mice. The amount of breast epithelial tissue was markedly decreased in *lats*$^{-/-}$ females, resulting in mammary fat pads, devoid of an epithelial component.

Figures 10A-E. Pituitary hyperplasia and dysfunction in *lats*$^{-/-}$ mice. (A and B) Histopathological sections of pituitaries derived from lats$^{-/+}$ (A) and *lats*$^{-/-}$ (B) mice. The paraffin sections were stained with hematoxylin and eosin. The normal pituitary gland from a *lats*$^{+/+}$ mouse demonstrates the organized architecture of the gland. Hyperplastic changes are visible in *lats*$^{-/-}$ pituitaries. Multiple atypical cells showing irregularly shaped nuclei, and variability in shape and size are readily apparent. (C-E) The graphs indicate the amount of LH (C), PRL (D), and FSH (E) production in the mice. Hormone levels are plotted as ng/ml with the results from the *lats*$^{+/-}$ mice represented by the right bar of each bar graph and the results from the *lats*$^{-/-}$ mice represented by the left bar of each bar graph.

Figures 11A-C. Soft tissue sarcoma development in *lats*$^{-/-}$ mice. (A,B) Typical soft tissue sarcomas in *lats*$^{-/-}$ mice (A, 6.5 months old; B, 4.5 months old). (C) Histopathological section of the soft tissue sarcoma shown in panel B stained with hematoxylin and eosin revealing pleiomorphic, spindle-shaped cells characteristic of this tumor.

Figure 12. Nucleotide and amino acid sequences of human lats (h-lats) (SEQ ID NOS:1 and 2, respectively).

Figure 13. Nucleotide and amino acid sequences of mouse lats (m-lats) (SEQ ID NOS:3 and 4, respectively).

Figure 14. Nucleotide and amino acid sequences of mouse lats2 (m-lats2) (SEQ ID NOS:5 and 6, respectively).

Figure 15. Nucleotide and amino acid sequences of *Drosophila* lats (SEQ ID NOS:7 and 8, respectively).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides lats-cdc2 protein complexes, including complexes that contain lats analogs and fragments and/or cdc2 analogs and fragments, as well as methods of producing these complexes and nucleic acids encoding the two members of the complex. The invention also provides antibodies that bind immunospecifically to a lats-cdc2 complex, but do not bind the individual binding partners immunospecifically.

The invention also provides methods for the modulation of cdc2 activity using lats proteins and lats analogs and derivatives that are able to interact with cdc2. In particular, methods are provided for treating or preventing diseases and disorders associated with aberrant cdc2 activity by administration of a therapeutic compound of the invention. The present invention further provides recombinant non-human animals, preferably mice, having at least one copy of (preferably both copies of, *i.e.,* is homozygous for) an inactivated *lats* gene, *i.e.*, *lats* knock-out animals. Preferably, these *lats* knock-out animals are generated by homologous recombination, *i.e.*, have a gene disrupted by insertional mutagenesis induced by homologous recombination with a nucleic acid containing non-lats sequences flanked by *lats* genomic sequences. The invention further provides methods of screening for compounds effective to treat or prevent cancer, preferably soft tissue sarcomas or ovarian tumors, more preferably skin cancer, using the recombinant non-human animals of the invention. The invention also provides methods of screening for compounds effective to treat or prevent pituitary dysfunction using the recombinant non-human animals of the invention.

Therapeutics of the invention that can be used to treat or prevent diseases and disorders associated with an aberrant level of cdc activity include those therapeutics that promote lats function (*e.g.*, lats proteins and lats derivatives and analogs that supply lats function, nucleic acids encoding lats, lats derivatives and analogs, and lats-cdc2 complexes), and those therapeutics that inhibit or antagonize lats function (*e.g.*, lats derivatives and analogs that inhibit or antagonize lats function) and nucleic acids encoding these lats derivatives and analogs, anti-lats antibodies and anti-lats-cdc2 complex antibodies, lats antisense nucleic acids, and lats inhibitors and antagonists.

The present invention also provides therapeutic methods and compositions for the treatment and prevention of cancer based on lats proteins and therapeutically or prophylactically effective analogs and fragments of lats proteins. The invention provides for treatment and prevention of cancer by administration of a therapeutic compound of the invention. The therapeutic compounds of the invention that can be used to treat or prevent cancer include: lats proteins, including human lats proteins, therapeutically or prophylactically effective lats analogs and fragments, and nucleic acids encoding the lats proteins, analogs and fragments.

In a preferred embodiment, the invention provides therapeutic and prophylactic methods for the treatment or prevention of cancer that has been shown to be or may be

refractory to chemotherapy or radiation therapy treatments or treatments based on tumor suppressor genes other than lats.

Also included in the invention are methods of screening lats proteins and lats derivatives and analogs for activity in treating cancer that has been shown to be or may be refractory to chemotherapy or radiation therapy. Additionally, the invention provides methods of screening lats proteins, lats derivatives and fragments, anti-lats antibodies, lats antisense nucleic acids, lats antagonists and inhibitors, and lats-cdc2 complexes for activity in modulating the activity of cdc2.

Therapeutic Uses

The invention provides for treatment or prevention of cancers refractory to chemotherapy or radiation therapy by administration of a therapeutic compound (termed herein "Therapeutic"). The invention also provides for treatment or prevention of diseases or disorders that can be treated by modulation of cdc2 activity by administration of a Therapeutic of the invention. Such "Therapeutics" include lats proteins and therapeutically or prophylactically effective analogs and fragments thereof; lats-cdc2 complexes; antibodies thereto; nucleic acids encoding the lats proteins, analogs, or fragments, and lats-cdc2 complexes; lats antisense nucleic acids, and lats agonists and antagonists .

In specific embodiments, the therapeutic is a lats protein or lats-cdc2 complex containing a lats protein that is phosphorylated, particularly a lats protein phosphorylated on a serine or threonine residue within 20 residues upstream of an Ala-Pro-Glu consensus in subdomain eight of a lats kinase domain, *e.g.*, a serine corresponding to serine 909 of human lats, as depicted in Figure 12 (SEQ ID NO:2). In another embodiment, the therapeutic is a lats derivative or lats-cdc2 complex containing a lats derivative, in which derivative a serine or threonine residue within 20 residues upstream of an Ala-Pro-Glu consensus in subdomain eight of a lats kinase domain is substituted with a glutamate or aspartate residue, preferably the serine corresponding to serine 909 of human lats is replaced with a glutamate residue. In a further embodiment, the therapeutic is a fragment of a lats protein or a lats-cdc2 complex containing a fragment of a lats protein comprising or consisting of the amino acid sequence of a lats protein corresponding to amino acids 15-585 of human lats, as depicted in Figure 12 (SEQ ID NO:2).

Generally, administration of products of a species origin or species reactivity (in the case of antibodies) that is the same species as that of the subject is preferred. Thus, in a preferred embodiment, a human lats protein, derivative, or fragment, or nucleic acid, or an antibody to a human lats protein, is therapeutically or prophylactically administered to a human patient.

Treatment and Prevention of Cancers Refractory to Chemotherapy or Radiotherapy

Cancers, including neoplasms, tumors, metastases, or any disorder characterized by uncontrolled cell growth, that have been shown to be refractory to a chemotherapy or

radiation therapy can be treated or prevented by administration of a Therapeutic of the invention that promotes (*i.e.*, increases or supplies) lats function.

Examples of such a Therapeutic include lats proteins, derivatives or fragments that are functionally active, particularly have a lats functional activity of inhibiting cell overproliferation (*e.g.*, as demonstrated in *in vitro* assays or in an animal model), and nucleic acids encoding a lats protein or a functionally active derivative or analog thereof (*e.g.*, for use in gene therapy). Other Therapeutics that can be used, *e.g.*, lats agonists, can be identified using *in vitro* assays or animal models, examples of which are described in Examples section.

That a cancer is refractory to chemotherapy or radiation therapy means that at least some significant portion of the cancer cells are not killed or their cell division arrested by the particular chemotherapeutic agent or combination of chemotherapeutic agents or the level of radiation employed in a therapeutic protocol. The determination of whether the cancer cells are refractory to the chemotherapy or the radiation therapy can be made either *in vivo* or *in vitro* by any method known in the art for assaying the effectiveness of treatment on cancer cells.

In various embodiments of the invention, cancer that is refractory to radiation therapy, chemotherapy or combination chemotherapy, or combination of radiotherapy and chemotherapy, is treated or prevented by administration of a Therapeutic of the invention. In a preferred embodiment, cancer that is refractory to treatment with a chemotherapeutic agent that is cell cycle specific, or said cancer is refractory to treatment with a chemotherapeutic agent that kills or arrests the cells in the S phase of the cell cycle, or said cancer is refractory to treatment with a chemotherapeutic agent that kills or arrests cells during the M phase of the cell cycle is treated using a Therapeutic of the invention. The Therapeutic of the invention can be administered along with radiation therapy and/or one or a combination of chemotherapeutic agents, or as an alternative to other forms of therapy.

The chemotherapy or radiation therapy administered concurrently with or subsequent to the administration of the therapeutic of the invention can be administered by any method known in the art. The chemotherapeutic agents are preferably administered in a series of sessions, any one or a combination of the chemotherapeutic agents listed above can be administered. With respect to radiation therapy, any radiation therapy protocol can be used depending upon the type of cancer to be treated. For example, but not by way of limitation, x-ray radiation can be administered; in particular, high-energy megavoltage (radiation of greater that 1 MeV energy) can be used for deep tumors, and electron beam and orthovoltage x-ray radiation can be used for skin cancers. Gamma ray emitting radioisotopes, such as radioactive isotopes of radium, cobalt and other elements may also be administered to expose tissues to radiation.

Malignancies

Malignancies and related disorders that may become refractory to chemotherapy and/or radiation therapy and that can be treated or prevented by administration of a Therapeutic that promotes lats function include blood-related cancers and solid tumors (for a review of such disorders, see Fishman et al., 1985, Medicine, 2d Ed., J.B. Lippincott Co., Philadelphia).

Treatment and Prevention of Diseases by Modulation of Cdc2 Activity

In a specific embodiment of the invention, diseases and disorders associated with aberrant levels of cdc2 activity, *e.g.*, aberrantly high or aberrantly low levels of cdc2 protein or activity, can be treated or prevented by administration of a therapeutic of the invention able to modulate the activity of cdc2. In particular, those diseases and disorders associated with an aberrantly high cdc2 activity are treated or prevented by administration of a Therapeutic that promotes lats activity. Alternatively, those diseases and disorders associated with an aberrantly low cdc2 activity are treated or prevented by administration of a Therapeutic that inhibits lats activity, *e.g.*, lats derivatives and analogs that inhibit or antagonize lats activity, anti-lats antibodies, lats antisense nucleic acids, lats inhibitors and antagonists, antibodies that specifically recognize a lats-cdc2 complex, etc.

Because cdc2 promotes cell division, diseases and disorders that may be associated with an increased level of cdc2 activity, as compared with the levels of cdc2 in a subject not afflicted with such a disease or disorder, include diseases and disorders associated with increased cell proliferation, such as malignancies. Diseases and disorders that may be associated with a decreased level of cdc2 activity include diseases and disorders associated with decreased cell proliferation.

Premalignant Conditions

The Therapeutics of the invention that reduce cdc2 activity can be administered to treat premalignant conditions and to prevent progression to a neoplastic or malignant state. Such prophylactic or therapeutic use is indicated in conditions known or suspected of preceding progression to neoplasia or cancer, in particular, where non-neoplastic cell growth consisting of hyperplasia, metaplasia, or most particularly, dysplasia has occurred.

Alternatively or in addition to the presence of abnormal cell growth characterized as hyperplasia, metaplasia, or dysplasia, the presence of one or more characteristics of a transformed phenotype, or of a malignant phenotype, displayed *in vivo* or displayed *in vitro* by a cell sample from a patient, can indicate the desirability of prophylactic/therapeutic administration of a Therapeutic that inhibits cdc2 activity. Some characteristics of a transformed phenotype include morphology changes, looser substratum attachment, loss of contact inhibition, loss of anchorage dependence, protease release, increased sugar transport, decreased serum requirement, expression of fetal antigens, disappearance of the 250,000 dalton cell surface protein, etc.

Treatment and Prevention of Disorders in Which Cell Proliferation Is Desired

Diseases and disorders involving a deficiency in cell proliferation (growth) or in which cell proliferation is otherwise desirable for treatment or prevention, are treated or prevented by administration of a Therapeutic that antagonizes (inhibits) lats function (in particular, lats-mediated inhibition of cell proliferation and/or lats binding to cdc2). Therapeutics that can be used include anti-lats antibodies (and fragments and derivatives thereof containing the binding region thereof), lats derivatives or fragments that are dominant-negative kinases, *lats* antisense nucleic acids, and *lats* nucleic acids that are dysfunctional (*e.g.*, due to a heterologous (non-lats sequence) insertion within the lats coding sequence) that are used to "knockout" endogenous lats function by homologous recombination (see, *e.g.*, Capecchi, 1989, Science 244:1288-1292), as described herein. Other Therapeutics that inhibit lats function can be identified by use of known convenient *in vitro* assays, *e.g.*, based on their ability to inhibit binding of lats to another protein (*e.g.*, cdc2), or inhibit any known lats function, as preferably assayed *in vitro* or in cell culture. Methods for screening for compounds that prevent or reduce lats binding to cdc2 are described herein. Preferably, suitable *in vitro* or *in vivo* assays are utilized to determine the effect of a specific Therapeutic (*i.e.*, its ability to promote cdc2 activity or increase cdc2 levels) and whether its administration is indicated for treatment of the affected tissue.

Diseases and disorders involving a deficiency in cell proliferation or in which cell proliferation is desired for treatment or prevention, and that can be treated or prevented by promoting cdc2 function, include degenerative disorders, growth deficiencies, hypoproliferative disorders, physical trauma, lesions, and wounds; for example, to promote wound healing, or to promote regeneration in degenerated, lesioned or injured tissues, etc.

Gene Therapy

Gene therapy refers to therapy performed by the administration of a nucleic acid to a subject. In this embodiment of the invention, the nucleic acid produces its encoded protein that mediates a therapeutic effect by promoting lats function.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel et al., 1993, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIBTECH 11(5):155-215. Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

In a preferred aspect, the Therapeutic comprises a *lats* nucleic acid that is part of an expression vector that expresses a lats protein or fragment or chimeric protein thereof in a

suitable host. In particular, such a nucleic acid has a promoter operably linked to the lats coding region, said promoter being inducible or constitutive, homologous or heterologous, and, optionally, tissue-specific. In another particular embodiment, a nucleic acid molecule is used in which the lats coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the *lats* nucleic acid, as described (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438). In another embodiment, a nucleic acid or combination of nucleic acids containing both a *lats* and a *cdc2* nucleic acid, preferably where each is operably linked to a promoter, is delivered by gene therapy methods.

Delivery of the nucleic acid into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vector, or indirect, in which case, cells are first transformed with the nucleic acid *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

Numerous techniques are known in the art for the introduction of foreign genes into cells (see *e.g.*, Loeffler and Behr, 1993, Meth. Enzymol. 217:599-618; Cohen et al., 1993, Meth. Enzymol. 217:618-644; Cline, 1985, Pharmac. Ther. 29:69-92).

In an embodiment in which recombinant cells are used in gene therapy, a *lats* nucleic acid or both *lats* and *cdc2* nucleic acids are introduced into the cells such that they are expressible by the cells or their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained *in vitro* can potentially be used, such as hematopoietic stem cells (HSC), stem cells of epithelial tissues such as the skin and the lining of the gut, embryonic heart muscle cells, liver stem cells (PCT Publication WO 94/08598, dated April 28, 1994), neural stem cells (Stemple and Anderson, 1992, Cell 71:973-985), or epithelial stem cells (ESCs) (Rheinwald, 1980, Meth. Cell Bio. 21A:229; Pittelkow and Scott, 1986, Mayo Clinic Proc. 61:771).

Antisense Therapy

Lats function may be inhibited by use of *lats* antisense nucleic acids. The present invention provides the therapeutic or prophylactic use of nucleic acids of at least six nucleotides and are preferably oligonucleotides (ranging from 6 to about 200 oligonucleotides), that are antisense to a gene or cDNA encoding a lats protein, or portions thereof. A *lats* "antisense" nucleic acid as used herein refers to a nucleic acid capable of hybridizing to a portion of a *lats* nucleic acid (preferably mRNA) by virtue of some sequence complementarity. The antisense nucleic acid may be complementary to a coding and/or noncoding region of a *lats* mRNA. In specific aspects, the oligonucleotide is at least 10 nucleotides, at least 15 nucleotides, at least 100 nucleotides, or at least 200 nucleotides.

The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at any position (examples of such modifications can be found in: Bailey, Ullmann's Encyclopedia of Industrial Chemistry (1998), 6th ed. Wiley and Sons). Such antisense nucleic acids have utility as Therapeutics that inhibit lats function or activity, and can be used in the treatment or prevention of disorders characterized by an aberrantly low cdc2 level or activity.

The *lats* antisense nucleic acids can be directly administered to a cell, or can be produced intracellularly by transcription of exogenous, introduced sequences. Alternatively, *lats* antisense nucleic acids are produced intracellularly by transcription from an exogenous sequence. For example, a vector can be introduced *in vivo* such that it is taken up by a cell, within which cell the vector or a portion thereof is transcribed, producing an antisense nucleic acid (RNA) of the invention. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art.

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a *lats* gene, preferably a human *lats* gene. However, absolute complementarity, although preferred, is not required.

Pharmaceutical compositions of the invention, comprising an effective amount of a *lats* antisense nucleic acid in a pharmaceutically acceptable carrier can be administered to a patient having a disease or disorder which is characterized by aberrantly low cdc2 activity.

The amount of *lats* antisense nucleic acid that will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. Where possible, it is desirable to determine the antisense cytotoxicity *in vitro*, and then in useful animal model systems prior to testing and use in humans.

In a specific embodiment, pharmaceutical compositions comprising *lats* antisense nucleic acids are administered via liposomes, microparticles, or microcapsules. In various embodiments of the invention, it may be useful to use such compositions to achieve sustained release of the *lats* antisense nucleic acids. In a specific embodiment, it may be desirable to utilize liposomes targeted via antibodies to specific identifiable central nervous system cell types (Leonetti et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87: 2448-2451; Renneisen et al., 1990, J. Biol. Chem. 265: 16337-16342).

Lats Proteins, Derivatives, Fragments and Lats-cdc2 Complexes

The lats proteins and nucleic acids, and lats derivatives and fragments can be produced by any method known in the art.

For recombinant expression of lats proteins, and lats derivatives and fragments, the nucleic acid containing all or a portion of the nucleotide sequence encoding the protein can

be inserted into an appropriate expression vector, *i.e.*, a vector that contains the necessary elements for the transcription and translation of the inserted protein coding sequence. In a preferred embodiment, the regulatory elements (*e.g.*, promoter) are heterologous (*i.e.*, not the native gene promoter). Promoters which may be used include the SV40 early promoter (Bernoist and Chambon, 1981, Nature 290: 304-310), and the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22: 787-797), among others.

A variety of host-vector systems may be utilized to express the protein coding sequence. These include mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.* baculovirus); microorganisms such as yeast containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA.

Once a lats protein, or derivative or fragment, has been recombinantly expressed, it may be isolated and purified by standard methods including chromatography (*e.g.*, ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. A lats protein may also be purified by any standard purification method from natural sources.

Alternatively, a lats protein, analog or derivative can be synthesized by standard chemical methods known in the art (*e.g.*, see Hunkapiller et al., 1984, Nature 310:105-111).

The Therapeutics of the invention also include derivatives and fragments related to lats. In particular embodiments, the derivative or fragment is functionally active, *i.e.*, capable of exhibiting one or more functional activities associated with a full-length, wild-type lats protein, *e.g.*, able to inhibit cell proliferation in *in vitro* and/or *in vivo* assays. Additionally, derivatives or fragments that inhibit lats activity, *e.g.*, promote cell proliferation, may also have a use in the methods of the invention. Derivatives or analogs of lats can be tested for the desired activity by procedures known in the art.

In specific embodiments of the invention, the Therapeutic is a lats protein that is phosphorylated, preferably that is phosphorylated on a serine or threonine residue within 20 amino acids upstream of an Ala-Pro-Glu consensus sequence in subdomain eight of a lats kinase domain, more preferably that is phosphorylated on a serine residue corresponding to serine 909 of human lats, as depicted in Figure 12 (SEQ ID NO:2). In another specific embodiment, the therapeutic is a lats derivative in which a serine or threonine residue within 20 residues upstream of an Ala-Pro-Glu consensus in subdomain eight of a lats kinase domain is substituted with a glutamate or aspartate residue, preferably, in which a serine residue corresponding to serine 909 of human lats is replaced with a glutamate residue. In another specific embodiment, the therapeutic is a fragment of a lats protein comprising or consisting of the amino acid sequence corresponding to amino acids 15 to 585 of human lats, as depicted in Figure 12 (SEQ ID NO:2).

In particular, lats derivatives can be made by altering lats sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Due

to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as a *lats* gene may be used in the practice of the present invention. These include nucleotide sequences comprising all or portions of *lats* genes which are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the lats derivatives of the invention include those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a lats protein including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

In a specific embodiment of the invention, proteins consisting of or comprising a fragment of a lats protein consisting of at least 10 (continuous) amino acids of the lats protein is provided. In other embodiments, the fragment consists of at least 20 or 50 amino acids of the lats protein. In specific embodiments, such fragments are not larger than 35, 100 or 200 amino acids. In a specific embodiment, the fragment of a lats protein is from the N-terminal portion of the protein, preferably including all or a portion of the amino acids corresponding to amino acids 15-585 of human lats. Derivatives or fragments of lats include but are not limited to those molecules comprising regions that are substantially homologous to lats or fragments thereof (*e.g.*, in various embodiments, at least 60% or 70% or 80% or 90% or 95% identity over an amino acid sequence of identical size with no insertions or deletions considered, or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art, *e.g.*, the blastp program) or whose encoding nucleic acid is capable of hybridizing to the inverse complement (the inverse complement of a nucleic acid strand has the complementary sequence running in reverse orientation to the strand so that the inverse complement would hybridize without mismatches to the nucleic acid strand; thus, for example, where the coding strand is hybridizable to a nucleic acid with no mismatches between the coding strand and the hybridizable strand, then the inverse complement of the hybridizable strand is identical to the coding strand) of a coding lats sequence, under high stringency, moderately stringency, or low stringency conditions, as discussed *infra*.

The lats derivatives and fragments of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at

the gene or protein level. For example, the cloned *lats* gene sequence can be modified by any of numerous strategies known in the art (Sambrook et al., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

Additionally, the lats-encoding nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem 253:6551), use of TAB® linkers (Pharmacia), etc.

Manipulations of the lats sequence may also be made at the protein level. Included within the scope of the invention are lats protein fragments or other derivatives which are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, etc. Any of numerous chemical modifications may be carried out by known techniques, including specific chemical cleavage by cyanogen bromide, trypsin, oxidation, reduction; etc.

In addition, analogs and fragments of lats can be chemically synthesized. For example, a peptide corresponding to a portion of a lats protein which comprises the desired domain, or which mediates the desired activity *in vitro*, can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the lats sequence.

In a specific embodiment, the lats derivative is a chimeric, or fusion, protein comprising a lats protein or fragment thereof (preferably consisting of at least a domain or motif of the lats protein, or at least 15, preferably 20, amino acids of the lats protein) joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different protein. In one embodiment, such a chimeric protein is produced by recombinant expression of a nucleic acid encoding the protein (comprising a lats-coding sequence joined in-frame to a coding sequence for a different protein). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer. Chimeric genes comprising portions of lats fused to any heterologous protein-encoding sequences may be constructed. A specific embodiment relates to a chimeric protein comprising a fragment of lats of at least six amino acids. In another specific embodiment, the lats derivative is a chimeric protein comprising a fragment of lats corresponding to amino acids 15-585 of human lats.

In another specific embodiment, the lats derivative is a molecule comprising a region of homology with a lats protein. By way of example, in various embodiments, a first

protein region can be considered "homologous" to a second protein region when the amino acid sequence of the first region is at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, or 95% identical, when compared to any sequence in the second region of an equal number of amino acids as the number contained in the first region with no insertions or deletions considered, or when compared to an aligned sequence of the second region that has been aligned by a computer homology program known in the art. For example, a molecule can comprise one or more regions homologous to a lats domain or a portion thereof.

Derivatives of Lats Containing One or More Domains of the Protein

In specific embodiments, the methods of the invention use lats derivatives and fragments that comprise, or alternatively consist of, one or more domains of a lats protein, including but not limited to a lats C-terminal domain 3 (LCD3), lats C-terminal domain 2 (LCD2), lats C-terminal domain 1 (LCD1), kinase domain, kinase subdomains, lats flanking domain (LFD) (amino-terminal to the kinase domain), lats split domain 1 (LSD1), lats split domain 2 (LSD2), SH3-binding domain, and opa repeat domain, functional (*e.g.*, binding) fragments of any of the foregoing, or any combination of the foregoing.

In human lats (h-lats), m-lats, m-lats2, and *Drosophila* lats, the LCD3 domain is the last three amino acids of the protein, which are Val-Tyr-Val in all four proteins. For human lats and *Drosophila* lats, the LCD2 domain is amino acid residues 1077-1086 and 1075-1084, respectively (all amino acid residues provided in this paragraph are for the human and *Drosophila* lats amino acid sequences depicted in Figures 12 and 14, respectively (SEQ ID NOS:2 and 8, respectively)); the LCD1 domain is amino acid residues 1032-1043 and 1035-1047, respectively; the kinase domain is amino acid residues 703-1014 and 711-1018, respectively; the LFD domain is amino acid residues 607-702 and 612-710 respectively; and the putative SH3-binding domain is amino acids 247-268 and 196-217, respectively. For the lats split domains in *Drosophila*, the LSD1 is amino acid residues 365-392 and the LSD2 is amino acids 536-544. In human lats, the LSD1 and LSD2 domains are split into anterior and posterior portions such that the LSD1 is amino acid residues 328-334 and 498-518 and LSD2 is amino acid residues 28-31 and 555-559.

In particular, the Therapeutics of the invention include molecules comprising specific fragments of lats that are those fragments in the respective lats protein most homologous to specific fragments of a human or mouse lats protein.

In a specific embodiment, a lats protein, derivative or fragment is provided that has a kinase domain and has a phosphorylated or dephosphorylated serine situated within 20 residues upstream of an Ala-Pro-Glu consensus in subdomain eight of its kinase domain, or in which the serine situated within 20 residues upstream of that consensus has been deleted or substituted by another amino acid. In various specific embodiments, the invention provides various phosphorylated and dephosphorylated forms of the lats protein, derivative, or fragment that are active or inactive kinase forms. Both phosphorylation and dephosphorylation of lats at different residues could potentially activate or inactivate lats.

Phosphorylation can be carried out by any methods known in the art, *e.g.*, by use of a kinase. Dephosphorylation can be carried out by use of any methods known in the art, *e.g.*, by use of a phosphatase.

Another specific embodiment relates to a derivative or fragment of a lats protein that is a dominant-active protein kinase. Such a derivative or analog comprises a lats kinase domain that has been mutated so as to be dominantly active (exhibit constitutively active kinase activity). It is known that acidic residues such as Glu and Asp sometimes mimic a phosphorylated residue, and changing the phosphorylatable Ser or Thr residue in subdomain eight into a Glu or Asp residue has been previously used to produce constitutively active kinases (Mansour et al., 1994, Science 265:966-970). Thus, changing a serine or threonine residue situated within 20 residues upstream of an Ala-Pro-Glu consensus in subdomain eight of a lats kinase domain into another residue (*e.g.*, Glu, Asp) may be used to make a dominant-active lats protein kinase. For example, changing Ser914 in *Drosophila* lats, or changing Ser909 in human lats, into a Glu residue could produce a dominant active lats kinase.

Another specific embodiment relates to a derivative or fragment of lats that is a dominant-negative protein kinase. Protein kinases can be mutated into dominant negative forms. Expression of a dominant negative protein kinase can suppress the activity of the wild-type form of the same kinase. Dominant negative forms of protein kinases are often obtained by expressing an inactive form of a kinase (Milarski and Saltiel, 1994, J. Biol. Chem. 269(33):21239-21243) or by expressing a noncatalytic domain of a kinase (Lu and Means, 1994, EMBO J. 12:2103-2113; Yarden et al., 1992, EMBO J. 11:2159-2166). Thus, a lats dominant-negative kinase can be obtained by mutating the kinase domain so as to be inactive (*e.g.*, by deletion and/or point mutation). For example, a lats derivative that is a dominant-negative kinase is a lats protein that lacks a kinase domain but comprises one or more of the other domains of the lats protein; *e.g.*, a lats protein derivative truncated at about the beginning of the kinase domain (*i.e.*, a lats fragment containing only sequences amino-terminal to the kinase domain). As another example, a lats derivative that is a dominant-negative kinase is a lats protein in which one of the residues conserved among serine/threonine kinases (see Hanks et al., 1988, Science 241:42-52) is mutated (deleted or substituted by a different residue).

In another specific embodiment, a molecule is provided that comprises one or more domains (or functional portion thereof) of a lats protein but that also lacks one or more domains (or functional portion thereof) of a lats protein. For example, such a protein may lack all or a portion of the kinase domain, but retain at least the SH3-binding domain of a lats protein. In another embodiment, a molecule is provided that comprises one or more domains (or functional portion thereof) of a lats protein, and that has one or more mutant (*e.g.*, due to deletion or point mutation(s)) domains of a lats protein (*e.g.*, such that the mutant domain has decreased function). The kinase domain may be mutant so as to have reduced, absent, or increased kinase activity.

Lats-cdc2 Complexes

The invention provides lats-cdc2 complexes. In a preferred embodiment, the lats-cdc2 complexes are complexes of human proteins. As used herein, fragment or derivative of a lats-cdc2 complex includes complexes where one or both members of the complex are fragments or derivatives of the wild-type lats or cdc2 protein. Such derivatives and fragments can be generated as described for lats derivatives and fragments above. Preferably, the lats-cdc2 complexes in which one or both members of the complex are a fragment or derivative of the wild type protein are functionally active lats-cdc2 complexes.

In particular aspects, the native proteins, derivatives or analogs of lats and/or cdc2 are of animals, *e.g.* mouse, rat, pig, cow, dog, monkey, human, fly, frog, or of plants. "Functionally active lats-cdc2 complex" as used herein refers to that material displaying one or more known functional attributes of a complex of full length lats with a full length cdc2, including but not exclusive to control of cell cycle progression, cell proliferation, etc.

In specific embodiments, the lats-cdc2 complex contains a lats protein that is phosphorylated, preferably that is phosphorylated on a serine or threonine residue within 20 amino acids upstream of an Ala-Pro-Glu consensus subdomain eight of a lats kinase domain, more preferably that is phosphorylated on a serine residue corresponding to serine 909 of human lats, as depicted in Figure 12 (SEQ ID NO:2). In another specific embodiment, the lats-cdc2 complex contains a lats derivative in which a serine or threonine residue within 20 residues upstream of an Ala-Pro-Glu consensus subdomain eight of a lats kinase domain is substituted with a glutamate or aspartate residue, preferably, in which a serine residue corresponding to serine 909 of human lats is replaced with a glutamate residue. In another specific embodiment, the therapeutic is a fragment of a lats protein comprising or consisting of the amino acid sequence corresponding to amino acids 15 to 585 of human lats, as depicted in Figure 12 (SEQ ID NO:2).

Methods are presented for screening lats-cdc2 complexes, as well as derivatives and fragments of the lats-cdc2 complexes for the ability to alter lats and/or cdc2 activity, *e.g.*, to alter cell proliferation. For example, such derivatives or fragments which have the desired immunogenicity or antigenicity can be used in immunoassays, for immunization, for inhibition of lats-cdc2 complex activity, etc. Derivatives or fragments that retain, or alternatively lack or inhibit, a property of interest (*e.g.*, participation in a lats-cdc2 complex) can be used as inducers, or inhibitors, respectively, of such a property and its physiological correlates. A specific embodiment relates to a lats-cdc2 complex of a fragment of lats and/or a fragment of cdc2 that can be bound by an anti-lats and/or anti-cdc2 antibody or antibody specific for a lats-cdc2 complex when such a fragment is included within a lats-cdc2 complex.

The lats-cdc2 complexes can be obtained by any method known in the art. The cdc2 nucleotide and amino acid sequence is available from GenBank, accession no. Y00272 (see also, Lee and Nurse, 1987, Nature 327:31-35). The lats-cdc2 complexes can be obtained, for example, by expressing an entire lats coding sequence and a cdc2 coding sequence in the

same cell, either under the control of the same promoter or two separate promoters. In yet another embodiment, a derivative, fragment or homolog of lats and/or a derivative, fragment or homolog of cdc2 are recombinantly expressed. Preferably, the derivative, fragment or homolog of lats and/or the cdc2 protein form a complex with a binding partner identified by a binding assay, such as co-immunoprecipitation with an anti-lats or anti-cdc2 antibody, or interaction in a yeast two-hybrid assay (Fields and Song, 1989, Nature 340:245-246; and Finley and Brent, in DNA Cloning 2, Rickwood and Hames, eds (Oxford University Press, Oxford, 1995)).

In a specific embodiment, fusion or chimeric proteins are provided that contain the domains of a lats protein, or, in a specific embodiment, the amino acid sequence corresponding to amino acids 15 to 585 of human lats, and a cdc2 protein that directly form a lats-cdc2 complex and, optionally, a heterofunctional reagent, such as a peptide linker, linking the two domains, where such a heterofunctional reagent, such as a reagent or linker promotes the interaction of the lats and cdc2 binding domains. These fusion proteins may be particularly useful where the stability of the interaction is desirable (due to the formation of the complex as an intramolecular reaction), for example in production of antibodies specific to the lats-cdc2 complex.

Generation of Antibodies to Lats Proteins and Lats-cdc2 Complexes and Derivatives

LATS proteins, including functional derivatives and fragments thereof (*e.g.* a LATS protein encoded by a sequence of any one of SEQ ID NOs:2, 4, 6, or 8, or a subsequence thereof) may be used as an immunogen to generate monoclonal or polyclonal antibodies and antibody fragments or derivatives (*e.g.*, chimeric, single chain, Fab fragments, etc.). For example, antibodies to a particular domain of a lats protein may be desired. In a specific embodiment, fragments of a lats protein identified as hydrophilic are used as immunogens for antibody production using art-known methods. Various known methods for antibody production can be used including cell culture of hybridomas; production of monoclonal antibodies in germ-free animals (PCT/US90/02545); the use of human hybridomas (Cole *et al.*, Proc. Natl. Acad. Sci. U.S.A. (1983) 80:2026-2030; Cole *et al.*, in Monoclonal Antibodies and Cancer Therapy (1985) Alan R. Liss, pp. 77-96), and production of humanized antibodies (Jones *et al.*, Nature (1986) 321:522-525; US Pat. No. 5,530,101).

Diagnostic, Prognostic, and Screening Uses of Lats-cdc2 Complexes

Lats-cdc2 complexes may be markers of specific disease states involving disruption of physiological processes, such as cell cycle progression and cell proliferation, and pathological processes, such as hyperproliferative disorders, including tumorigenesis and tumor progression, and hypoproliferative disorders, and thus have diagnostic utility.

Detecting levels of lats-cdc2 complexes, or individual lats and cdc2 proteins or the mRNA encoding lats and cdc2 may be used in diagnosis or prognosis, to follow the course of disease states, or to follow therapeutic response, etc.

Lats-cdc2 complexes, lats and cdc2 proteins, and derivatives, and sub-sequences thereof, *lats* and/or *cdc2* nucleic acids (and sequences complementary thereto), and anti-lats-cdc2 complex antibodies and combinations of antibodies directed against lats and cdc2 have uses in diagnostics. Such molecules can be used in assays, such as immunoassays, to detect, prognose, diagnose, or monitor various conditions, diseases, and disorders characterized by aberrant levels of lats-cdc2 complexes or monitor the treatment thereof.

In particular, such an immunoassay is carried out by a method comprising contacting a sample derived from a patient with an anti-lats-cdc2 complex antibody under conditions such that immunospecific binding can occur, and detecting or measuring the amount of any immunospecific binding by the antibody. In a specific aspect, such binding of antibody, in tissue sections, can be used to detect aberrant lats-cdc2 complex localization or aberrant (*e.g.*, high, low or absent) levels of lats-cdc2 complex. In a specific embodiment, an antibody to a lats-cdc2 complex can be used to assay in a patient tissue or serum sample for the presence of a lats-cdc2 complex where an aberrant level of lats-cdc2 complex is an indication of a diseased condition. By "aberrant levels" is meant an increased or decreased level relative to that present, or a standard level representing that present, in an analogous sample from a portion of the body or from a subject not having the disorder.

The immunoassays which can be used include competitive and non-competitive assay systems using techniques such as Western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, etc.

Nucleic acids encoding lats and cdc2 proteins and related nucleotide sequences and sub-sequences, including complementary sequences, can also be used in hybridization assays. The *lats* and *cdc2* nucleotide sequences, or sub-sequences thereof comprising about at least 8 nucleotides, can be used as hybridization probes. Hybridization assays can be used to detect, prognose, diagnose, or monitor conditions, disorders, or disease states associated with aberrant levels of the mRNAs encoding the components of a lats-cdc2 complex. In particular, such a hybridization assay is carried out by a method comprising contacting a sample containing nucleic acid with a nucleic acid probe capable of hybridizing to *lats* and *cdc2* DNAs or RNAs, under conditions such that hybridization can occur, and detecting or measuring any resulting hybridization.

By way of example, levels of lats-cdc2 complexes and lats and cdc2 proteins can be detected by immunoassay, levels of lats and cdc2 mRNA can be detected by hybridization assays (*e.g.*, Northern blots, dot blots), binding of lats to cdc2 can be done by binding assays commonly known in the art, translocations and point mutations in *lats* and/or *cdc2* can be detected by Southern blotting, RFLP analysis, PCR using primers that preferably

generate a fragment spanning at least most of the *lats* and/or *cdc2* gene, sequencing of the *lats* and/or *cdc2* genomic DNA or cDNA obtained from the patient, etc.

Also embodied are methods to detect a lats-cdc2 complex in cell culture models that express a lats-cdc2 complex, or derivatives thereof, for the purpose of characterizing or preparing the lats-cdc2 complex for harvest. This embodiment includes cell sorting of prokaryotes such as but not restricted to, bacteria (Davey and Kell, 1996, Microbiol. Rev. 60:641-696), primary cultures and tissue specimens from eukaryotes, including mammalian species such as human (Steele et al., 1996, Clin. Obstet. Gynecol 39:801-813), and continuous cell cultures (Orfao and Ruiz-Arguelles, 1996, Clin. Biochem. 29:5-9).

Kits for diagnostic use are also provided, that comprise in one or more containers an anti-lats-cdc2 complex antibody and, optionally, a labeled binding partner to the antibody. Alternatively, the anti-lats-cdc2 complex antibody can be labeled (with a detectable marker, *e.g.*, a chemiluminescent, enzymatic, fluorescent, or radioactive moiety). A kit is also provided that comprises in one or more containers a nucleic acid probe or probes capable of hybridizing to *lats* and *cdc2* mRNAs. In a specific embodiment, a kit can comprise in one or more containers a pair of primers (*e.g.*, each in the size range of 6-30 nucleotides) that are capable of priming amplification [*e.g.*, by polymerase chain reaction (see *e.g.*, Innis et al., 1990, PCR Protocols, Academic Press, Inc., San Diego, CA), ligase chain reaction (see EP 320,308) use of ß-replicase, cyclic probe reaction, or other methods known in the art], under appropriate reaction conditions of at least a portion of a *lats* nucleic acid and a *cdc2* nucleic acid. A kit can optionally further comprise in a container a predetermined amount of a purified lats-cdc2 complex, lats and cdc2 proteins or nucleic acids thereof, *e.g.*, for use as a standard or control.

Demonstration of Therapeutic Utility

The Therapeutics of the invention are preferably tested *in vitro*, and then *in vivo*, for the desired therapeutic or prophylactic activity, prior to use in humans. For example, *in vitro* assays which can be used to determine whether administration of a specific Therapeutic is indicated, include *in vitro* cell culture assays in which a patient tissue sample is grown in culture, and exposed to or otherwise administered a Therapeutic, and the effect of such Therapeutic upon the tissue sample is observed. A lower level of proliferation or survival of the contacted cells indicates that the Therapeutic is effective to treat the condition in the patient. Alternatively, instead of culturing cells from a patient, Therapeutics may be screened using cells of a tumor or malignant cell line. Many assays standard in the art can be used to assess such survival and/or growth; for example, cell proliferation can be assayed by measuring ^{3}H-thymidine incorporation, by direct cell count, by detecting changes in transcriptional activity of known genes such as proto-oncogenes (*e.g.*, fos, myc) or cell cycle markers; cell viability can be assessed by trypan blue staining, differentiation can be assessed visually based on changes in morphology, etc.

In another embodiment of the invention, a Therapeutic of the invention is screened for activity to modulate (*e.g.*, promote, inhibit or antagonize) cdc2 levels and/or activity. The levels of cdc2 protein and mRNA and cdc2 activity can be determined by any method well known in the art. For example, cdc2 protein can be quantitated by known immunodiagnostic methods such as western blotting immunoprecipitation using any antibody against cdc2 (for example, anti-cdc2 antibodies are commercially available from Santa Cruz Inc.) Cdc2 mRNA can be quantitated by methods that are well known and routine in the art, for example by northern analysis, RNase protection, the polymerase chain reaction in connection with the reverse transcription, etc. Cdc2 activity can also be assayed by any method known in the art, for example, by the histone-H1 kinase assay.

Compounds for use in therapy can be tested in suitable animal model systems prior to testing in humans, including but not limited to in rats, mice, chicken, cows, monkeys, rabbits, etc.

In a preferred embodiment, *lats* knock-out mice, *e.g.*, as described in the Examples, are used to test therapeutics of the invention for activity to treat or prevent cancers, or to modulate cdc2 activity.

***Lats* Knock-out Animals**

The invention provides recombinant non-human animals in which one or more *lats* genes have been inactivated, *e.g.*, "knock-out animals". The recombinant non-human animal can be any animal, *e.g.*, mouse, rats, rodents, hamster, sheep, pig, cow, *Drosophila*, *C. elegans*, insects, worms, primates, dogs, etc., and is preferably a mouse. Such an animal can be generated by any method known in the art for disrupting a gene on the chromosome of an animal. *Lats* knock-out animals do not include animals in which one or more *lats* genes have been inactivated by naturally occurring mutations. In a preferred aspect, a *lats* knock-out animal can be produced by promoting homologous recombination between a *lats* gene in its chromosome and an exogenous *lats* gene that has been rendered biologically inactive (preferably by insertion of a heterologous sequence, *e.g.*, an antibiotic resistance gene). Homologous recombination methods for disrupting genes in the mouse genome are described, for example, in Capecchi (1989, Science 244:1288-1292) and Mansour et al. (1988, Nature 336:348-352). A *lats* knock-out mouse may be produced by the method described in the Examples section.

Briefly, all or a portion of a *lats* genomic clone is isolated from genomic DNA from the same species as the knock-out animal. The *lats* genomic clone can be isolated by any method known in the art for isolation of genomic clones (*e.g.*, by probing a genomic library with a probe derived from a lats sequence, such as those sequences provided in Figures 12-15, *i.e.*, SEQ ID NOS:1, 3, 5, or 7). Once the genomic clone is isolated, all or a portion of the clone is introduced into a recombinant vector. Preferably, the portion of the clone introduced into the vector that contains at least a portion of an exon of the *lats* gene, *i.e.*, contains a lats protein coding sequence. A sequence not homologous to the *lats* sequence,

preferably a positive selectable marker, such as a gene encoding an antibiotic resistance gene, is then introduced into the *lats* gene exon. The selectable marker is preferably operably linked to a promoter, more preferably a constitutive promoter. The non-homologous sequence is introduced anywhere in the lats coding sequence that will disrupt lats activity, *e.g.*, at a position where point mutations or other mutations have been demonstrated to inactivate lats protein function. For example, the non-homologous sequence can be inserted for the coding sequence for the portion of the lats protein containing all or a portion of the kinase domain (*e.g.*, the nucleotide sequence coding for at least 50, 100, 150, 200 or 250 amino acids of the kinase domain), the Lats C-terminal domain 1, the Lats C-terminal domain 2, and the Lats C-terminal domain 3, or, more preferably, for the sequence coding for the amino acids corresponding to 756 to 1130 of human lats (as depicted in Figure 12 (SEQ ID NO:2) and as indicated in the alignment of human and mouse lats in Figure 6A).

The positive selectable marker is preferably a neomycin resistance gene (neo gene) or a hygromycin resistance gene (hygro gene). The promoter may be any promoter known in the art; by way of example the promoter may be the phosphoglycerate kinase (PKG) promoter (Adra et al.,1987, Gene 60:65-74), the PolII promoter (Soriano et al., 1991. Cell 64:693-701), or the MC1 promoter, which is a synthetic promoter designed for expression in embryo-derived stem cells (Thomas & Capecchi, 1987, Cell 51:503-512). Use of a selectable marker, such as an antibiotic resistance gene, allows for the selection of cells that have incorporated the targeting vector (for example, the expression of the neo gene product confers resistance to G418, and expression of the hygro gene product confers resistance to hygromycin).

In a preferred embodiment, a negative selectable marker for a counterselection step for homologous, as opposed to non-homologous, recombination of the vector is inserted outside of the *lats* genomic clone insert, *e.g.*, as shown in Figure 6B. For example, such a negative selectable marker is the HSV thymidine kinase gene (HSV-tk), the expression of which makes cells sensitive to ganciclovir. The negative selectable marker is preferably under the control of a promoter such as the PGK promoter, the PolII promoter or the MC1 promoter.

When homologous recombination occurs, the portions of the vector that are homologous to the *lats* gene, as well as the non-homologous insert within the *lats* gene sequences, are incorporated into the *lats* gene in the chromosome, and the remainder of the vector is lost. Thus, since the negative selectable marker is outside the region of homology with the *lats* gene, cells in which homologous recombination has occurred (or their progeny), will not contain the negative selectable marker. For example, if the negative selectable marker is the HSV-tk gene, the cells in which homologous recombination has occurred will not express thymidine kinase and will survive exposure to ganciclovir. This procedure permits the selection of cells in which homologous recombination has occurred, as compared to non-homologous recombination in which it is likely that the negative

selectable marker is also incorporated into the genome along with the lats sequences and the positive selectable marker. Thus, cells in which non-homologous recombination has occurred would most likely express thymidine kinase and be sensitive to ganciclovir.

Once the targeting vector is prepared, it is linearized with a restriction enzyme for which there is a unique site in the targeting vector, and the linearized vector is introduced into embryo-derived stem (ES) cells (Gossler et al., 1986, Proc. Natl. Acad. Sci. USA 83:9065-9069) by any method known in the art, for example by electroporation. If the targeting vector includes a positive selectable marker and a negative, counterselectable marker, the ES cells in which homologous recombination has occurred can be selected by incubation in selective media. For example, if the selectable markers are the neo resistance gene and the HSV-tk gene, the cells are exposed to G418 (*e.g.*, approximately 300 μg/ml) and ganciclovir (*e.g.*, approximately 2 μM).

Any technique known in the art for genotyping, for example Southern blot analysis or the polymerase chain reaction, can be used to confirm that the disrupted lats sequences have homologously recombined into the *lats* gene in the genome of the ES cells. Because the restriction map of the *lats* genomic clone is known (see Figure 6b) and the sequence of the lats coding sequence is known (see Figure 13), the size of a particular restriction fragment or a PCR amplification product generated from DNA from both the disrupted and non-disrupted alleles can be determined. Thus, by assaying for a restriction fragment or PCR product, the size of which differs between the disrupted and non-disrupted *lats* gene, one can determine whether homologous recombination has occurred to disrupt the *lats* gene.

The ES cells with the disrupted lats locus can then be introduced into mouse blastocysts by microinjection and then the blastocysts can be implanted into the uteri of pseudopregnant mice using routine techniques. The mice that develop from the implanted blastocysts are chimeric for the disrupted allele. The chimeric male mice can be crossed to female mice, and this cross can be designed such that germline transmission of the allele is linked to transmission of a certain coat color. The germline transmission of the allele can be confirmed by Southern blotting or PCR analysis, as described above, of genomic DNA isolated from tail samples.

Isolating *Lats* Genes

Clones comprising lats nucleotide sequences, particularly *lats* genomic clones, can be isolated by any method known in the art. The nucleotide sequences encoding, and the corresponding amino acid sequences of, human lats, mouse lats, mouse lats2 and *Drosophila* lats are provided in Figures 12-15, respectively (SEQ ID NOS:1-8, respectively) and bacterial cells containing the plasmid pBS(KS)-h-lats, which contains the gene encoding human lats, were deposited on March 24, 1995 with the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2201, and assigned Accession No. 69769. *Lats* nucleic acids, either *lats* genomic clones or *lats* specific probes to identify *lats* genomic clones, can be obtained by any method known in the art, *e.g.*, from

the deposited plasmid, by the polymerase chain reaction (PCR) using synthetic primers hybridizable to the 3' and 5' ends of a lats nucleotide sequence and/or by cloning from a cDNA or genomic library using an oligonucleotide probe specific for the gene sequence, such as a probe from the *lats* gene insert in plasmid pBS(KS)-h-lats. Genomic clones can be identified by probing a genomic DNA library under appropriate hybridization conditions, *e.g.*, high stringency conditions, low stringency conditions or moderate stringency conditions, depending on the relatedness of the probe to the genomic DNA being probed. For example, if the lats probe and the genomic DNA are from the same species, then high stringency hybridization conditions may be used; however, if the lats probe and the genomic DNA are from different species, then low stringency hybridization conditions may be used. High, low and moderate stringency conditions are all well known in the art.

Procedures for low stringency hybridization are as follows (see also Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. USA 78:6789-6792): Filters containing DNA are pretreated for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 μg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 μg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 106 cpm ^{32}P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 hours at 40°C, and then washed for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 hours at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and reexposed to film.

Procedures for high stringency hybridizations are as follows: Prehybridization of filters containing DNA is carried out for 8 hours to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 μg/ml denatured salmon sperm DNA. Filters are hybridized for 48 hours at 65°C in prehybridization mixture containing 100 μg/ml denatured salmon sperm DNA and 5-20 X 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for 1 hour in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1 X SSC at 50°C for 45 minutes before autoradiography.

Moderate stringency conditions for hybridization are as follows: Filters containing DNA are pretreated for 6 hours at 55°C in a solution containing 6X SSC, 5X Denhardt's solution, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution and 5-20 X 10 cmp ^{32}P-labeled probe is used. Filters are incubated in the hybridization mixture for 18-20 hours at 55°C, and then washed twice for 30 minutes at 60°C in a solution containing 1 X SSC and 0.1% SDS.

Any eukaryotic cell potentially can serve as the nucleic acid source for the molecular cloning of the *lats* gene. The nucleic acid sequences encoding lats can be isolated from vertebrate, mammalian, human, porcine, bovine, feline, avian, equine, canine, as well as

additional primate sources, insects, etc. The DNA may be obtained by standard procedures known in the art, preferably from cloned genomic DNA (*e.g.*, a DNA "library") from the desired cell (see, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II.). The gene should be molecularly cloned into a suitable vector for propagation of the gene.

In preferred embodiments, the genomic clone used to generate a recombinant, non-human animal by homologous recombination contains at least a portion of the lats coding sequence of SEQ ID NO:3; alternatively, the genomic clone contains at least a portion of the lats coding sequence of SEQ ID NO:5.

Methods of Screening Therapeutics Using *Lats* Knock-out Mice

The invention provides methods for screening for compounds useful in the treatment or prevention of cancer or in the treatment and prevention of pituitary diseases and disorders by administration or application of the compound to be tested to a *lats* knock-out animal, preferably a *lats* knock-out mouse.

In a preferred embodiment, the invention provides a method for screening a potential therapeutic compound for activity in treating or preventing cancer. The potential therapeutic compound is administered to a recombinant non-human animal having at least one inactivated *lats* gene (*i.e.*, a *lats* knock-out animal, preferably a *lats* knock-out mouse), preferably two inactivated lats gene *lats* genes (*i.e.*, is homozygous for the inactivated *lats* allele). The size or progression of the cancer is then compared to that before the compound was added, or to a comparable recombinant animal without the administration of the compound, or to a normal, non-recombinant animal. A decrease in the size or progression of the cancer in the recombinant non-human animal after the administration of the compound as compared to the same animal prior to the administration or to another recombinant non-human animal not so administered or the standard size or progression of the cancer indicates that the compound has activity in treating or preventing cancer

The screening method of the invention can be used to screen for potential therapeutic compounds for the treatment or prevention of any cancer, preferably a cancer or neoplastic disease that is caused by the *lats* knock-out mutation. As described in the Examples section, *infra*, *lats* knock-out mice are susceptible to ovarian stromal tumors and soft tissue sarcomas that metastasize to vital organs. Accordingly, in preferred embodiments, the invention provides methods for screening compounds useful in treating or preventing ovarian tumors and soft tissue sarcomas. *Lats* knock-out mutations in other animals or in other *lats* homologs may make the resulting knock-out animal susceptible to other types of neoplastic disease. The invention also contemplates use of these other *lats* knock-out animals to screen compounds for efficacy in treating or preventing the types of neoplastic diseases found in these *lats* knock-out animals. Additionally, compounds

effective to treat or prevent ovarian tumors and/or soft tissue sarcomas in *lats* knock-out animals may also be effective to treat or prevent other types of cancers and neoplastic disease. Thus, *lats* knock-out animals may be used to screen for compounds that have activity to treat or prevent these other types of cancers and neoplastic disease.

The invention also provides methods of screening compounds for efficacy in treating or preventing skin cancer. As demonstrated in Examples section, *infra*, exposure to carcinogens induced, at a high frequency, skin tumors in the *lats* knock-out mice. Many methods are known in the art for inducing skin carcinogenesis in animals (for review see DiGiovanni, 1992, Pharmac. Ther. 54:63-128). Generally, mouse skin tumors can be elicited by application of a carcinogenic dose of tumor initiator, *e.g.*, 600 to 800 nmole of a pure polycyclic aromatic hydrocarbon such as 9,10-dimethyl-1,2-benzanthracene (DMBA). Other tumor initiators include, but are not limited to, arylamines, carbamates, haloalkylethers, haloaromatics, lactones, nitro-aromatics, nitrosamides and ureas. Alternatively, and preferably, mouse skin tumors can be induced by an initial application of a single sub-carcinogenic dose of a tumor initiator, *e.g.*, DMBA, and then repeated doses or exposures to a tumor promoter, such as phorbolesters (*e.g.*, TPA), teleocidins, polyacetates, okadaic acid, calyculin A, palytoxin, and thapsigargin. Ultraviolet B (UVB) radiation, skin abrasion and skin wounding are also strong tumor promoters.

In a preferred embodiment, such skin tumors are induced by a two-step process comprising a single treatment with DMBA, preferably 50 µl of a 0.5% DMBA solution in acetone, to the dorsal surface of the mouse 1 to 5 days after birth followed by repeated exposure to UVB irradiation, *e.g.*, exposures of approximately three times per week with an initial exposure of approximately 100 mJ/cm^2 per session, increasing the dosage by 10% per treatment (unless erythema or scaling occurs) to a maximum of 700 mJ/cm2, with an average of about 27 treatment sessions per mouse (Serrano et al., 1996, Cell 85:27-37).

Accordingly, in a preferred embodiment, the invention provides a method for screening a potential therapeutic compound for activity in treating or preventing skin cancer comprising administering the compound to a recombinant non-human animal in which one, preferably two, *lats* genes have been inactivated (i.e., a *lats* knock-out animal) and in which recombinant non-human animal tumors have been induced by exposure to at lease one carcinogen. The size or progression of the skin tumors are then compared before and after the administration of the compound. A reduction in the size or progression of the skin tumors in the recombinant non-human animal administered the compound as compared to same animal prior to administration of the compound or to the animal not so administered or to the standard size or progression of the skin tumors, indicates that the compound has activity in treating or preventing skin cancer. In another embodiment, the compound to be screened is administered by recombinantly expressing the compound in the recombinant non-human animal inactivated for the *lats* gene.

The administration of the compound to be tested can be carried out by any method known in the art, *e.g.*, orally, intravenously, intramuscularly, intraperitoneally,

subcutaneously, rectally, topically, etc. For the screening of compounds for efficacy in treating or preventing skin cancer, the compound is preferably applied topically.

After administration of the compound to be tested, the tumors, sarcomas, and other cancers can be evaluated by any diagnostic or histopathological method for detecting and evaluating tumors and cancers, for example, by visual inspection of the tumors (particularly for skin tumors), manual palpitation of tumors, biopsy or surgical removal of the tumor tissue and subsequent inspection, and sacrifice and dissection of the recombinant non-human animal. Morphological evaluation of tissue, either removed by biopsy or dissected from a sacrificed mouse, may be performed by fixing the tissue by any method known in the art, for example, in 10% neutral buffered formalin at 4°C, and subsequent dehydration, *e.g.*, in ethanol. The fixed and dehydrated tissue may be embedded in paraffin and then sectioned, for example into 4-5 mm sections by any method known in the art. Sections can be stained, for example, with a standard stain, such as hematoxylin and eosin, for microscopic inspection.

Another aspect of the invention provides methods for screening potential therapeutic compounds for efficacy in treating or preventing diseases or disorders associated with pituitary dysfunction. *Lats* knock-out mice display a number of consequences of pituitary dysfunction, as described in the Examples section, *infra*. The methods of the invention can be used to screen compounds for efficacy in treating or preventing such pituitary dysfunctions as pituitary hyperplasia, fertility defects, such as defective ovulation, lack of breast development, abnormal reproductive cycles in females, LH hypogonadotropic hypogonadism, reduced levels of pituitary hormones, specifically LH, GH and PRL, and reduced growth and metabolic abnormalities caused by reduced GH levels. Therapeutics that are effective to treat one or more of these conditions associated with pituitary dysfunction may also be effective to treat or prevent other conditions, diseases or disorders associated with pituitary dysfunction.

In a preferred embodiment, potential therapeutic compounds to be screened for activity in treating or preventing diseases and disorders associated with pituitary dysfunction are administered to a recombinant non-human animal in which one or more chromosomal copies of the *lats* gene have been inactivated (*i.e.*, a *lats* knock-out animal, preferably and *lats* knock-out mouse). Levels of an indicator of pituitary function or dysfunction are then compared in the recombinant non-human animal before and after the compound was administered. In one embodiment, the compound to be screened is administered by recombinantly expressing the compound in the recombinant non-human animal having an inactivated *lats* gene.

Indicators of pituitary function that may be assayed include fertility, ovulation, the female reproductive cycle (*e.g.*, the estrus cycle), breast tissue development, growth or size of the animal, including weight, skeletal size, *e.g.*, of the skull and/or longitudinal bones, and organ weight, and serum levels of LH, GH and PRL. These indicators may be measured by any means known in the art for evaluating these indicators. For example,

fertility may be evaluated by attempting to mate an animal and determining whether conception occurred, measuring sperm count in male animals or detecting ovulation in female animals. The reproductive organ tissue may also be examined histopathologically (*e.g.*, by fixing, sectioning and staining the tissue for inspection) for morphological defects, particularly in the testis, ovaries, and breast tissue. Whether the animal goes through an estrus cycle may be determined by observation of the animal. Hormone levels may be determined by any method known in the art, for example in serum samples by radio immunoassay using antibodies specific for the particular hormone. Lack of normal growth can be determined by measuring the animal *e.g.*, the weight, size of the skull and/or longitudinal bones, or organ weight, during maturation.

Candidate Therapeutics

Candidate therapeutics may come from any source of therapeutics known in the art. For example, these therapeutics can be proteins, nucleic acids (including anti-sense nucleic acids), antibodies, peptides, organic molecules, etc. In some instances, compounds may be screened first in *in vitro* assays to determine their potential as anti-cancer or anti-pituitary dysfunction therapeutics.

For example, chemical libraries may be screened for useful therapeutics. Exemplary libraries are commercially available from several sources (ArQule, Tripos/PanLabs, ChemDesign, Pharmacopoeia). Many diversity libraries suitable for use are known in the art and can be used to provide compounds to be tested according to the present invention. Alternatively, libraries can be constructed using standard methods. Chemical (synthetic) libraries (Houghten et al., 1991, Nature 354:84-86; Lam et al., 1991, Nature 354:82-84; Medynski, 1994, Bio/Technology 12:709-710; Gallop et al., 1994, J. Medicinal Chemistry 37(9):1233-1251), recombinant expression libraries, or polysome-based libraries are exemplary types of libraries that can be used. Other examples include combinatorial libraries (Ohlmeyer et al., 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926; Erb et al., 1994, Proc. Natl. Acad. Sci. USA 91:11422-11426; Houghten et al., 1992, Biotechniques 13:412; Jayawickreme et al., 1994, Proc. Natl. Acad. Sci. USA 91:1614-1618; Salmon et al., 1993, Proc. Natl. Acad. Sci. USA 90:11708-11712), organic diversity (*e.g.*, nonpeptide) libraries (Bunin et al., 1994, Proc. Natl. Acad. Sci. USA 91:4708-4712) may be used. Libraries of non-peptides, *e.g.*, peptide derivatives (for example, that contain one or more non-naturally occurring amino acids) can also be used. One example of these are peptoid libraries (Simon et al., 1992, Proc. Natl. Acad. Sci. USA 89:9367-9371). Peptoids are polymers of non-natural amino acids that have naturally occurring side chains attached not to the alpha carbon but to the backbone amino nitrogen. Since peptoids are not easily degraded by human digestive enzymes, they are advantageously more easily adaptable to drug use.

Therapeutic/Prophylactic Administration and Compositions

The invention provides methods of treatment (and prophylaxis) by administration to a subject of an effective amount of a Therapeutic of the invention. In a preferred aspect, the Therapeutic is substantially purified. The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human. In a specific embodiment, a non-human mammal is the subject.

Various delivery systems are known and can be used to administer a Therapeutic of the invention, *e.g.*, encapsulation in liposomes (Langer, Science 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989)), microparticles, microcapsules, recombinant cells capable of expressing the Therapeutic, receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), construction of a Therapeutic nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local.

In a specific embodiment where the Therapeutic is a nucleic acid encoding a protein Therapeutic, the nucleic acid can be administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), etc. Alternatively, a nucleic acid Therapeutic can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a Therapeutic, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the Therapeutic is administered. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the Therapeutic, preferably in purified form, together with a suitable amount of carrier so as to

provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection.

The amount of the Therapeutic of the invention which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. However, suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention.

Screening for Lats Agonists and Antagonists

Lats nucleic acids, proteins, and derivatives may be used in screening assays to detect molecules that specifically bind to lats nucleic acids, proteins, or derivatives and thus have potential use as agonists or antagonists of lats, in particular, molecules that thus affect cell proliferation and/or cdc2 activity, or molecules that promote or inhibit formation of lats-cdc2 complexes. In a preferred embodiment, such assays are performed to screen for molecules with potential utility as lead compounds for drug development, particularly as anti-cancer drugs. The invention thus provides assays to detect molecules that specifically bind to lats nucleic acids, proteins, or derivatives or bind to or interfere with the formation of lats-cdc2 complexes. For example, recombinant cells expressing *lats* nucleic acids can be used to recombinantly produce lats proteins in these assays, to screen for molecules that bind to a lats protein, and recombinant cells expressing *lats* and *cdc2* nucleic acids can be used to recombinant produce both lats and cdc2 proteins in these assays, to screen for molecules that bind to or inhibit formation of a lats-cdc2 complex. Molecules (*e.g.*, putative binding partners of lats) are contacted with the lats protein (or fragment thereof) under conditions conducive to binding, and then molecules that specifically bind to the lats protein or bind to or interfere with the formation of lats-cdc2 complexes are identified.

Similar methods can be used to screen for molecules that bind to lats derivatives or nucleic acids. Methods that can be used to carry out the foregoing are commonly known in the art.

By way of example, diversity libraries, such as random or combinatorial peptide or nonpeptide libraries can be screened for molecules that specifically bind to lats. Many libraries are known in the art that can be used, *e.g.*, chemically synthesized libraries, recombinant (*e.g.*, phage display libraries), and *in vitro* translation-based libraries.

Examples of chemically synthesized libraries are described in Fodor et al., 1991, Science 251:767-773; Houghten et al., 1991, Nature 354:84-86; Lam et al., 1991, Nature 354:82-84; Medynski, 1994, Bio/Technology 12:709-710; Gallop et al., 1994, J. Medicinal Chemistry 37(9):1233-1251; Ohlmeyer et al., 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926; Erb et al., 1994, Proc. Natl. Acad. Sci. USA 91:11422-11426; Houghten et al., 1992, Biotechniques 13:412; Jayawickreme et al., 1994, Proc. Natl. Acad. Sci. USA 91:1614-1618; Salmon et al., 1993, Proc. Natl. Acad. Sci. USA 90:11708-11712; PCT Publication No. WO 93/20242; and Brenner and Lerner, 1992, Proc. Natl. Acad. Sci. USA 89:5381-5383.

Examples of phage display libraries are described in Scott and Smith, 1990, Science 249:386-390; Devlin et al., 1990, Science, 249:404-406; Christian, R.B., et al., 1992, J. Mol. Biol. 227:711-718; Lenstra, 1992, J. Immunol. Meth. 152:149-157; Kay et al., 1993, Gene 128:59-65; and PCT Publication No. WO 94/18318 dated August 18, 1994.

In vitro translation-based libraries include but are not limited to those described in PCT Publication No. WO 91/05058 dated April 18, 1991; and Mattheakis et al., 1994, Proc. Natl. Acad. Sci. USA 91:9022-9026.

By way of examples of nonpeptide libraries, a benzodiazepine library (see *e.g.*, Bunin et al., 1994, Proc. Natl. Acad. Sci. USA 91:4708-4712) can be adapted for use. Peptoid libraries (Simon et al., 1992, Proc. Natl. Acad. Sci. USA 89:9367-9371) can also be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh et al. (1994, Proc. Natl. Acad. Sci. USA 91:11138-11142).

Screening the libraries can be accomplished by any of a variety of commonly known methods. See, *e.g.*, the following references, which disclose screening of peptide libraries: Parmley and Smith, 1989, Adv. Exp. Med. Biol. 251:215-218; Scott and Smith, 1990, Science 249:386-390; Fowlkes et al., 1992; BioTechniques 13:422-427; Oldenburg et al., 1992, Proc. Natl. Acad. Sci. USA 89:5393-5397; Yu et al., 1994, Cell 76:933-945; Staudt et al., 1988, Science 241:577-580; Bock et al., 1992, Nature 355:564-566; Tuerk et al., 1992, Proc. Natl. Acad. Sci. USA 89:6988-6992; Ellington et al., 1992, Nature 355:850-852; U.S. Patent No. 5,096,815, U.S. Patent No. 5,223,409, and U.S. Patent No. 5,198,346, all to Ladner et al.; Rebar and Pabo, 1993, Science 263:671-673; and PCT Publication No. WO 94/18318.

In a specific embodiment, screening can be carried out by contacting the library members with a lats protein (or nucleic acid or derivative) immobilized on a solid phase and

harvesting those library members that bind to the protein (or nucleic acid or derivative). Examples of such screening methods, termed "panning" techniques are described by way of example in Parmley and Smith, 1988, Gene 73:305-318; Fowlkes et al., 1992, BioTechniques 13:422-427; PCT Publication No. WO 94/18318; and in references cited hereinabove.

In another embodiment, the two-hybrid system for selecting interacting proteins in yeast (Fields and Song, 1989, Nature 340:245-246; Chien et al., 1991, Proc. Natl. Acad. Sci. USA 88:9578-9582) can be used to identify molecules that specifically bind to a lats protein or derivative or that interfere with the formation of lats-cdc2 complexes. Additionally, the two hybrid system or co-immunoprecipitation of lats and cdc2 can be used as assays to screen for compounds that promote or inhibit formation of lats-cdc2 complexes.

In a preferred embodiment, the invention provides a method of screening for a molecule that modulates (*i.e.*, inhibits, antagonizes or promotes) directly or indirectly, the formation of a complex of lats and cdc2 proteins comprising measuring the levels of said complex formed from lats and cdc2 proteins in the presence of said molecule (optionally, purified) under conditions conducive to formation of the complex; and comparing the levels of said complex with the levels of said complex that are formed in the absence of said molecule, lower or higher level of said complex in the presence of said molecule indicates that the molecule modulates formation of said complex.

EXAMPLES

EXAMPLE 1: Human Lats Modulates Cdc2/Cyclin A Activity

Using mammalian cell culture assays, we have found that lats is phosphorylated in a cell cycle-dependent manner and that it complexes with cdc2 in early mitosis. Lats associated cdc2 has no mitotic cyclin partner and no kinase activity for histone H1. Furthermore, we have found that *lats* mutant cells in *Drosophila* abnormally accumulate cyclin A. These biochemical observations indicate that lats is a negative regulator of cdc2/cyclin A, a finding supported by *in vivo* genetic data demonstrating that lats specifically interacts with cdc2 and cyclin A in *Drosophila*.

Materials and Methods

For yeast two-hybrid experiments, DNA encoding N-terminal h-lats (amino acid numbers 15 to 585 of the human lats protein sequence as depicted in Figure 12 (SEQ ID NO:2)), C-terminal h-lats (amino acid number 589 to 1130 of the human lats protein sequence as depicted in Figure 12 (SEQ ID NO:2)), and h-lats (amino acid numbers 15 to 1130 of the human lats protein sequence as depicted in Figure 12 (SEQ ID NO:2)) were cloned into pBTM116 (Bartel et al., Cellular Interactions in Development: A Practical Approach, ed. D. Hartley (Oxford University Press, Oxford, England (1993)). DNA

encoding human cdc2, CDK2, CDK4 and C-terminal h-lats were cloned in pACT (Durfee et al., 1993, Genes & Devel. 8:440-452). The constructs were transformed into yeast strain L40 (Vojtek et al., 1993, Cell 74:205-214). The transformants were tested for growth on SD his-ura-trp-leu medium (Bio101, California) and for ß-galactosidase activities (Vojtek et al., 1993, Cell 74:205-214).

For baculovirus experiments, full-length *h-lats* cDNA was cloned into the vector pBacPAK8 and baculovirus were produced according to the protocols provided by Clontech. IPLB-Sf21 cells were co-infected with equal amounts of h-lats and human cdc2-baculoviruses and were harvested 62 hours after infection for immunoprecipitation and immunoblot assay.

Fly Genetics

The full-length *h-lats* cDNA was cloned into the vector pCaSpeR-hs (Tummel and Pirrott, 1992, *Drosophila* Information Service 71:150). Multiple transformant lines were obtained and used in rescue experiments with $lats^{e532}$, $lats^{e26\text{-}1}$, $lats^{a1}$, and $lats^{X1}$ alleles. Expression of *hs-h-lats* was induced as described in Xu et al. (1995, Development 121:1053-1063) -- incubation at 37°C for one hour every day until eclosure. Since the induction of *h-lats* requires heat-shock treatments, X-ray irradiation was used to induce mitotic clones in *y w P[hs-h-lats]/y w; P[FRT]82B* $lats^{x\text{-}1}$*/P[y+)96E* animals. The rest of the *lats* mutant mitotic clones were induced and labeled according to Xu and Rubin (1993, Development 117:1223-1237). The *Drosophila lats* and *h-lats* cDNAs were cloned into the pGMR vector to generate multiple transformant lines (Hay et al., 1994, Development 120:2121-2129). Besides the mutations mentioned above, $cdc2c^{E136E}$ (a gift of Helena Richardson), $cycA^{neo114}$, *Df(2R)59A-B* were used for *cdc2c*, *cyclin A* and *cyclin B*, respectively.

Tissue Culture

HeLa cells were synchronized at different cell cycle stages by various treatments as described by Knehr et al.(1995, Exp. Cell. Res. 217:546-553). Briefly, cells were arrested at G1 by thymidine and hydroxyurea treatment; at S phase by thymidine double block (incubation in the presence of 2 mM thymidine for 24 hours, followed by recovery in the absence of thymidine for 12 hours, followed by another incubation in 2 mM thymidine for 14 hours) plus a 4 hour incubation in medium without thymidine; and at G2 by thymidine double block plus an 8 hour incubation in medium without thymidine. To arrest cells in M phase, cells were treated with 0.1 μg/ml nocodazole (Sigma) for 12 hours and mitotic cells were shaken off of the flask and washed twice with cold DMEM without serum. These mitotic cells were then resuspended in fresh warm medium without nocodazole and incubated in suspension at 37°C. Cells were harvested at various time points after removal of nocodazole (herein "ARN") for further analysis. CHO cells were grown in a-MEM medium plus 7% FBS and IPLB-Sf21 cells were grown in sf-900 II SFM plus 10% FBS.

Antibodies and Immunochemistry

Anti-human lats rat monoclonal and rabbit polyclonal antibodies were raised against a GST-N-h-Lats (GST fused to the N-terminal portion of lats, *i.e.*, consisting of amino acids 15-585 of the human lats amino acid sequence as depicted in Figure 12 (SEQ ID NO:2) fusion protein. Anti-human cdc2 (#sc 054), anti-human cyclin B (#sc 245), anti-human cyclin A (#sc 239) monoclonal antibodies were purchased from Santa Cruz Inc. Rabbit polyclonal anti-*Drosophila* Cyclin A and B antibodies were gifts of David Glover. Monoclonal mouse anti-BrdU antibodies (#347580) were purchased from Becton Dickinson and monoclonal mouse ant-c-myc antibodies (#OP 10) were purchased from Oncogene Sciences. Propidium iodide (Sigma) was used as a DNA marker.

HeLa, CHO, or IPLB Sf21 cells were lysed in TG buffer (1% Triton, 10% glycerol) (Sun et al., 1996, Genes & Devel. 10:395-406) plus freshly added proteinase inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 2 μg/ml aprotinin). The lysates were centrifuged at 50 K, 4°C for 12 minutes. Supernatants were pre-cleaned by incubating with protein G-agarose. Immunoprecipitation and western blots were performed by the procedures described by Sun et al. (1996, Genes & Devel. 10:395-406). Western blots were visualized by enhanced chemiluminescence (Amersham). Whenever necessary the blots were stripped following the procedure described by Edgar et al. (1994, Genes & Devel. 8:440-452). Calf Intestinal Phosphatase (CIP) treatments were carried out as described in Sun et al. (1996, Genes & Devel. 10:395-406).

H1 Kinase Assay

HeLa cell lysates (50 minutes ARN) were precleaned by incubation in protein G-agarose. Immunoprecipitates were washed three times with TG buffer and twice with 1X kinase buffer (50 mM Tris-HCl 7.5, 10 mM MgCl2, 5 mM EGTA, 2 mM DTT) without DDT. The kinase assay was carried out on ice for 10 minutes in 35 μl of 1X kinase buffer containing 15 μCi of γ-^{32}P ATP, 1.6 μg of histone H1, and 1.5 μM ATP. The kinase activities were measured by quantifying the intensities of histone-H1 phosphorylation using a PhosphorImager (Molecular Dynamics). The amounts of cdc2 in the immunoprecipitates were determined by anti-cdc2 immunoblotting and densitometer scanning (Molecular Dynamics). The kinase assay experiments were repeated three times.

Results

Human Lats Can Functionally Replace its Fly Counterpart

Sequence conservation suggested that human lats could be a functional homolog of *Drosophila* lats. To test this, the human *lats* cDNA was introduced into the *Drosophila* genome under the control of the heat shock-inducible promoter (*hs-h-lats*) (Lis et al., 1983, Cell 35:403-410) and expressed the transgene was expressed under the conditions previously established for rescue using the fly *lats* gene (Xu et al., 1995, Development 121:1053-1064; and PCT Publication WO 96/30402, published October 3, 1996). In

mosaic flies, clones of cells mutant for *lats* undergo extensive overproliferation and develop into large tumors in various tissues (Figure 1A and Xu et al., 1995, Development 121:1053-1064). Expression of human lats completely suppressed tumor formation in *lats* mosaic flies (Figures 1B-D). Instead, the *lats* mutant cells (genetically marked as *yellow*- cells) in these human lats-expressing mosaic animals developed into normal adult structures (Figures 1C and D). The ability of the human gene to support normal fly development was further examined. Expression of the human *lats* transgene in homozygous *lats* mutant *Drosophila* rescued all developmental defects including embryonic lethality found in homozygous *lats* mutants. Furthermore, the extent of phenotypic rescue correlated with the level of human lats expression. Complete phenotypic rescue required daily induction of human lats, and leaky expression of lats controlled by the heat shock promoter at 25°C resulted in partial suppression of the *lats* mutant overproliferation phenotype (Figures 1E and F). These data demonstrate that human lats is an authentic homolog of the *Drosophila* lats tumor suppressor.

Lats is phosphorylated in a cell cycle-dependent manner

To further explore the function of lats, the biochemical properties of the human lats protein were examined. Lats immunoprecipitated from HeLa cells had two major migrating forms (Figure 2A, lane 6). The slow-migrating form of lats was converted into the fast-migrating form after the proteins were incubated with calf intestine alkaline phosphatase (CIP) (Figure 2A). Addition of a phosphatase specific inhibitor, ß-glycerophosphate, to the phosphatase reaction blocked this conversion (Figure 2A, lanes 5 and 10). These results indicate that the slow-migrating form is phosphorylated lats, while the fast-migrating form is dephosphorylated lats.

Lats immunoprecipitated from cells at different mitotic stages displayed varying amounts of the two forms (compare lanes 1 and 6 of Figure 2A), suggesting that the phosphorylation state of lats may oscillate with the cell-cycle. To verify this possibility, lats proteins were immunoprecipitated from extracts of HeLa cells at G0, G1, S, and G2 phases, and different time points during mitosis (minutes after removal of nocodazole (ARN) block) (Knehr, et al., 1995, Exp. Cell Res. 217:546-553). DAPI staining was used to verify the cell cycle progression. All lats protein was phosphorylated at late prophase (0 minutes ARN; Figure 2B), and remained phosphorylated through metaphase (50 minutes ARN; Figures 2B and C). Dephosphorylated lats could be detected when cells in the culture begin to enter anaphase (75 minutes ARN; Figures 2B and C), and by the start of telophase (100 minutes ARN) most of the lats protein was dephosphorylated (Figures 2B and C). In late mitosis, G1, S, G2 or G0 phase, lats molecules were in the dephosphorylated form (Figure 2B). These observations strongly suggest that the lats protein undergoes two major phosphorylation changes during the cell cycle. At the G2/M boundary or in early prophase, lats is phosphorylated, and lats becomes dephosphorylated at the metaphase/anaphase boundary or in early anaphase.

Lats complexes with cdc2 during mitosis

Lats mutant cells in *Drosophila* mosaic for the *lats* mutation do differentiate, indicating that mutations in *lats* do not block cellular differentiation in general (Figures 1G and H). The *lats* mutant overproliferation phenotype and cell cycle-dependent phosphorylation of lats suggest that the protein could be directly involved in the regulation of the cell cycle.

Immunoprecipitation experiments were carried out to examine whether lats proteins complex with known cell cycle regulators. Interestingly, cdc2 was found to co-immunoprecipitate with lats in mitotic cells (Figures 3A and B). The co-precipitation of lats and cdc2 was confirmed in both murine and human cells using several polyclonal and monoclonal anti-human lats antibodies (as described above in the Materials and Methods section, *supra*). Although similar amounts of lats were immunoprecipitated from HeLa cells during different stages of mitosis (Figure 3B, upper panel), the amount of co-precipitated cdc2 varied (Figure 3B, lower panel). Co-precipitated cdc2 was most abundant at early mitosis (0 and 50 minutes ARN; Figure 3B; also see Figures 2B and C for cell cycle progression). The amount of co-precipitated cdc2 then progressively decreased as the cell cycle progressed (Figure 3B--lanes 100', 150', and 200'). No cdc2 co-immunoprecipitation could be detected in quiescent cells in G0 (Figure 3A and Figure 2B). The difference in the amount of co-precipitated cdc2 cannot be attributed to changes of cdc2 levels during the cell cycle, since it has previously been shown that the cdc2 protein is maintained at a nearly constant level in cycling cells (Dalton, 1992, EMBO J. 11:1797-1804; McGowan et al., 1990, Mol. Cell. Biol. 10:3847-3851). Indeed, equal amounts of cdc2 protein were precipitated from the 50 minutes and 200 minutes ARN extracts when anti-cdc2 antibodies were used.

The interaction of lats and cdc2 proteins was studied by expressing human lats and cdc2 proteins in the baculovirus expression system. The baculovirus-expressed cdc2 and lats proteins could be co-immunoprecipitated using either anti-human lats or anti-cdc2 antibodies (Figure 3C). This result suggests that the *in vivo* lats/cdc2 complex may result from direct binding of the two proteins.

The interaction between lats and cdc2 was also examined using the yeast two-hybrid assay (Fields and Song, 1989, Nature 340:245-245; Finley and Brent, in DNA Cloning 2, Rickwood and Hames, eds (Oxford University Press (Oxford, 1995)). Consistent with the co-immunoprecipitation results, full length lats and the N-terminal region of lats interacted with cdc2 in the assay (Figure 3E). Since the C-terminal kinase domain of lats did not interact with cdc2, these results indicate that lats associates with cdc2 through its N-terminal domain. Furthermore, neither full length lats nor the N-terminal region of lats showed any interaction with two G1 cell cycle kinases, CDK2 and CDK4 (Figure 3E), indicating that the association between lats and cdc2 is specific.

The lats/cdc2 complex is inactive for H1 kinase activity

We examined whether the lats/cdc2 complex has any kinase activity on the substrate histone H1. Lats/cdc2 and cdc2/cyclin B complexes were immunoprecipitated separately from 50 minute ARN HeLa cell extracts using either anti-human lats or anti-cyclin B monoclonal antibodies and assayed for histone H1 kinase activities. In contrast to the cdc2/cyclin B complex, the lats/cdc2 complex showed no detectable kinase activity for histone H1 (Figure 3D). Densitometer readings indicated that the H1 kinase activity of the lats/cdc2 complex does not differ from the background control and is at least 25 fold lower than the kinase activity of the cdc2/cyclin B complex. These results indicate that cdc2 molecules associated with lats are inactive or have dramatically reduced mitotic kinase activity.

The lack of H1 kinase activity in the lats-associated cdc2 could be due to the inhibition of the kinase activity of the cdc2/cyclin complex by lats. Alternatively, the lats/cdc2 complex may lack cyclin A and B which are the indispensable subunits for cdc2 kinase activity (Draetta et al., 1989, Cell 56:829-838; Solomon et al., 1990, Cell 63:1013-1024). Neither cyclin A nor cyclin B proteins could be detected in the lats/cdc2 immunocomplex when probed with anti-cyclin A and B antibodies (Figure 3B), indicating that lats modulates cdc2 activity in a way different from that of the known cyclin dependent kinase inhibitors (CDIs) (Sherr, 1996, Science 274:1672-1677; Harper, 1997, Cancer Surveys 29:91-107).

Lats genetically interacts with cdc2 and cyclin A during *Drosophila* development

In *Drosophila*, cdc2 also complexes with cyclin A or B (Knoblich et al., 1994, Cell 77:107-120). We examined the potential genetic interactions between lats, cdc2, cyclin A and cyclin B in *Drosophila*. Animals heterozygous for the strong *cdc2* allele, $cdc2^{B47}$, or homozygous for the temperature sensitive $cdc2^{ts}$ mutation at permissive temperature are viable and morphologically normal (Clegg et al., 1993, Genome 36:676-685). The $lats^{P8}$ mutation causes late pupal lethality in homozygous mutants (Figure 4A), and reducing cdc2 activity in $lats^{P8}$ homozygotes by introducing one copy of a *cdc2* mutant allele ($cdc2^{B47}$ or $cdc2^{ts}/+$; $lats^{P8}/lats^{P8}$) was sufficient to rescue the lats-associated lethality (Figure 4B). Furthermore, the overproliferation phenotype of $lats^{P8}$ adult appendages were also suppressed. Rescued animals had near-wild type eyes in comparison to the overproliferated, large, rough eyes of the $lats^{P8}$ mutants (Figures 4C and D). Reducing cdc2 activity also suppressed the giant larvae/pupae and disc overproliferation phenotypes of the $lats^{E26-1}$ animals (Figures 4E and F). The *Drosophila* CDK2 homolog, cdc2c complexes with Cyclin E (Lehner and O'Farrell, 1990, Cell 61:535-547; Knoblich et al., 1994, Cell 77:107-120). Consistent with the result of the yeast two-hybrid assay for human lats and CDK2 (Figure 3C), inactivation of one copy of the *cdc2* gene did not modify the phenotypes of the $lats^{P8}$ mutant animals. We further examined the potential genetic

interaction between *lats*, *cyclin A* and *cyclin B* in *Drosophila* as described above. Interestingly, while cyclin B did not interact with lats, cyclin A behaved similarly to cdc2. Inactivation of one copy of the cyclin A gene resulted in almost identical phenotypic suppression of the $lats^{P8}$ and $lats^{E26-1}$ mutants as did the *cdc2* mutants (data not shown). Thus, the specific genetic interactions between lats, cdc2, and cyclin A confirms the biochemical data indicating that lats regulates cell proliferation by negatively modulating cdc2/cyclin A activity.

While cdc2 protein remains at a constant level during the cell cycle (Dalton, 1992, EMBO J. 11:1797-1804; McGowan et al., 1990, Mol. Cell. Biol. 10:3847-3851), cyclin A and B are degraded when the cdc2/cyclin complexes are inactivated (Draetta et al., 1989, Cell 56:829-828; Murray et al., 1989, Nature 339:280-286; King et al., 1994, Cell 79:563-571).

Accordingly, whether lats inactivation leads to accumulation of cyclin A and B was also determined. In the *Drosophila* eye imaginal disc, cyclin A and B are detected in cells anterior to the morphogenetic furrow (MF) as well as in a stripe of cells posterior to the MF which are undergoing the last round of cell division (the second mitotic wave) (Thomas et al., 1994, Cell 77:1003-1014). In clones of lats cells located anterior to the MF, anti-cyclin A or B antibody staining did not detect any obvious changes in levels of the two proteins (Figures 5A-N; Whitfield et al., 1990, EMBO J. 9:2563-2572), which might be due to the fact that cells in this region already accumulate high levels of both cyclin A and B. Cells in the MF are synchronized in G1 and so are the cells posterior to the MF which are differentiating into neurons. Both populations of the G1 cells have no detectable cyclin A or B (Thomas et al., 1994, Cell 77:1003-1014; Figures 5B and H). However, in clones of $lats^{+}$ cells in the MF and in the region posterior to the MF, a high level of cyclin A was detected (Figures 5A-E). Interestingly, in $lats^{-/-}$ cells, cyclin A was still degraded in cells at late mitosis (Figure 5F), indicating that lats affects limited aspects of the cell cycle. Finally, consistent with the genetic interaction results, $lats^{-/-}$ clones did not cause obvious changes in the staining pattern of cyclin B (Figures 5G and H). These results provide direct evidence indicating that inactivation of *lats* causes overproliferation by deregulating cdc2/cyclin A activity.

The role of lats in cell cycle regulation was examined by overexpressing lats in the developing eye imaginal disc. When *Drosophila* and human *lats* cDNAs were expressed in cells in and posterior to the MF under the direction of the GMR promoter (Hay et al., 1994, Development 120:2121-2129), they exhibited similar phenotypes. Adult eyes from these animals were smaller than wild type and had irregular architecture with missing bristles, a phenotype reminiscent to that of overexpressing p21 under the same promoter (*GMR-p21*; de Nooij and Hariharan, 1995, Science 270:983-985) (Figures 5I and J). Sections of GMR-lats adult retinas also revealed a phenotype identical to that observed in retinas from flies transformed with *GMR-p21*. While almost all of the ommatidia contained the full complement of photoreceptor cells, many pigment cells were missing (Figure 5K). This

phenotype suggested that, like overexpression of p21, overexpression of lats blocked the last round of cell division in the developing eye disc. Further examination of the GMR-lats eye discs revealed that in the region of second mitotic wave there was an accumulation of cells with intense propidium-iodide staining, indicating that the cells were tetraploid, which was followed immediately posteriorly by apoptotic cells with fragmented nuclei (Figure 5L). In contrast to the overexpression of p21 which blocked entry into S phase, overexpressing lats arrested cells at G2/M or M phase. Consistent with this conclusion, BrdU labeling experiments revealed that the S phase of the cells in the second mitotic wave did occur in GMR-lats eye discs (Figures 5M and N).

Discussion

The lats molecules are a novel family of conserved proteins

Expression of human lats under the same conditions used for rescue by the fly gene completely suppressed tumor formation in *lats* mosaic flies and rescued all developmental defects in *lats* homozygous mutant *Drosophila*. These experiments provide definitive evidence for functional conservation among the *lats* genes, indicating that human lats can perform all functions that are normally provided by the fly protein.

A model for lats function

Our biochemical and genetic data support the hypothesis that lats is a negative regulator of cdc2/cyclin A. Cdc2 co-immunoprecipitated with lats using either HeLa and CHO cell extracts or baculovirus-expressed proteins. Cdc2 also interacted with lats in yeast two-hybrid assays. Moreover, lats-associated cdc2 has no cyclin A or B subunit and no histone H1 kinase activity. In *Drosophila*, *lats* mutant cells abnormally accumulated cyclin A. Genetic data in *Drosophila* demonstrate that the overproliferation and lethality phenotypes of *lats* mutants can be suppressed by mutations in *cdc2* and *cyclin A* genes. The genetic interaction between lats, cdc2, and cyclin A is highly specific. While animals heterozygous for cdc2 or cyclin A did not display any defects, removal of one copy of either gene was sufficient to dominantly suppress the *lats* mutant phenotypes. Such a genetic interaction was not observed in hundreds of genes examined, including other positive cell cycle regulators such as *cdc2c*, *cyclin B*, and *dE2F*.

The lats kinase domain contains all 11 subdomains previously found in other protein kinases (Hanks et al., 1988, Science 241:42-45), suggesting that it is an active protein kinase. However, lats alone and lats/cdc2 complex do not appear to have any autophosphorylation activity or phosphorylation activity for cdc2 and histone H1. Yeast two-hybrid experiments showed that the N-terminal region of lats interacted with cdc2 much more strongly than did full-length lats (Figure 3E). This result indicates that the C-terminal kinase domain of lats has a negative effect on the binding between the lats N-terminal region and cdc2.

The association of lats with cdc2 is directly correlated with its state of phosphorylation (compare Figures 2B and 3B). During early mitosis, lats is phosphorylated and associates with cdc2. At G0, lats is dephosphorylated and fails to associate with cdc2. Furthermore, the transition of the lats phosphorylation state during mitosis correlates with a change in its ability to bind to cdc2. Phosphorylation is a common mechanism that regulates protein activities during the cell cycle (Hunter, 1995, Cell 80:225-236).

Cell-cell communication mechanism regulating cell proliferation

Many tumor suppressors probably evolved to play important regulatory roles during development. The study of the normal developmental functions of a tumor suppressor is essential to our understanding of the mechanisms of tumorigenesis. A growing body of evidence suggests that proliferating cells in a developing *Drosophila* imaginal disc communicate to maintain a constant disc size, and that lats plays an important role in this process. Using mutations such as *Minute* and *dE2F* in *Drosophila*, it has been shown that, from a young mosaic disc containing cells of different genotypes, the number of progeny cells from a given parental cell can vary dramatically in a mature disc, while the overall size of the mature disc is unaffected (Simpson, 1979, Devel. Biol. 69:182-193; Simpson and Morata, 1981, Devel. Biol. 85:299-308; Brook et al., 1996, EMBO J. 15:3676-3683). Imaginal discs can also undergo regeneration when a small region of a disc is surgically removed, a phenomenon similar to liver regeneration in mammals (French et al., 1976, Science 193:969-981; Meinhardt, 1994, Bioessays 16:627-632; Michalopoulos and DeFrances, 1997, Science 276:60-66). Consistent with the notion that proliferation is regulated by local cell interaction, it has been shown that DNA replication and mitosis in growing discs occur in small, non-clonal clusters of cells throughout the disc (Adler and MacQueen, 1981, Exp. Cell Res. 133:452-456; Milan et al., 1996, Proc. Natl. Acad. Sci. USA 93:11687-11692; Milan et al., 1996, Proc. Natl. Acad. Sci. USA 93:640-645). Furthermore, young discs transplanted into adult hosts grow until the disc reaches its normal size, indicating that such a size control mechanism is an intrinsic property of the cells in each disc (Bryant in The Genetics and Biology of *Drosophila*, Vol. 2c, Ashburner and Novitski, eds. (Academic Press, New York, 1978). Transplantation experiments in mice revealed a similar size control phenomenon with anlagen of some vertebrate organs (Leitina et al., 1971, Transplantation 11:499-502).

Lats mutations dramatically disrupt the size and shape of discs in *Drosophila*. Clones of *lats* mutant cells in mosaic discs overproliferate to form massive outgrowths that are sometimes larger than the mature discs themselves, and animals homozygous for many *lats* alleles also have dramatically overgrown discs (Figures 1A and F; Xu et al., 1995, Development 121:1053-1063). These *lats* phenotypes indicate that an inhibitory cell-cell communication mechanism has been disrupted and suggest that the lats protein could be a component of this mechanism regulating cell proliferation. The overproliferation phenotype of lats behaves in a cell autonomous fashion: inactivating lats causes mutant cells

to overproliferate (Xu et al., 1995, Development 121:1053-1063). Furthermore, in mosaic discs containing *lats* mutant clones, there is an overproliferation of *lats* mutant cells as well as a reduction in the number of wild type cell. These observations are consistent with a regulatory mechanism where *lats* mutant cells are able to send signals inhibiting cell proliferation but are defective in receiving such signals.

While the mammalian cdc2/cyclin A complex is involved in G2/M regulation (Hamaguchi et al., 1992, J. Cell Biol. 117:1041-1053; Hunter and Pines, 1994, Cell 79:573-582), *Drosophila* cdc2/cyclin A functions at the G1/S phase transition in addition to the G2/M phase transition. Ectopic activation of cdc2/cyclin A by overexpressing cyclin A in G1 arrested cells can drive the G1/S transition and induce S phase in cells lacking cyclin E (Dong et al., 1997, Genes & Devel. 11:94-105; Sprenger et al., 1997, Curr. Biol. 7:488-499). This G1/S activity is greatly enhanced when both cyclin A and an activated form of cdc2 are overexpressed. In *roughex (rux)* mutants, cells accumulate cyclin A in early G1 and progress into S phase precociously (Thomas et al., 1994, Cell 77:1003-1014; Thomas et al., 1997, Genes & Devel. 11:1289-1298). Loss of fizzy-related (fzr), a cdc2-related fly gene, results in accumulation of mitotic cyclins in G1 cells and causes progression through an extra division cycle in the embryonic epidermis (Sigrist and Lehner, 1997, Cell 90:671-681). These observations have shown that in *Drosophila* extra cdc2/Cyclin A activity can cause overproliferation. Consistent with these observations, we find that Cyclin A is abnormally accumulated in *lats* mutant cells (Figures 5A-N) and *lats* phenotypes can be suppressed by *cdc2* and *cyclin A* mutations. Several aspects of the *lats* phenotype are unique. First, *lats* mutants deregulate cdc2/cyclin A activities which affects both the G1/S and G2/M transitions. Second, while mutants such as *rux, fizzy(fzy)*, and *fzr* accumulate multiple mitotic cyclins and thus affect activities of several cdc2/cyclin complexes (Thomas et al., 1994, Cell 77:1003-1014; Dawson et al., 1995, J. Cell Biol. 129:725-737; Sigrist and Lehner, 1997, Cell 90:671-681), *lats* mutants appear to only affect cdc2/cyclin A. Finally, in *lats* mutant cells, cyclin A is degraded at late mitosis (Figure 5F), further indicating that many aspects of the cell cycle are normal in *lats* mutants. These properties distinguish *lats* mutants from genetic alterations that affect multiple CDK/cyclin complexes or that abnormally activate CDK/cyclin at a single cell-cycle stage or throughout the entire cell cycle, and provide an explanation for the extensive overproliferation phenotype of the *Drosophila lats* mutants.

The data provided herein indicate that cdc2/cyclin A activity is negatively regulated by the lats protein. Yeast two-hybrid assays show that lats specifically interacts with cdc2 but not other CDKs. Genetic data in *Drosophila* also show that lats interacts with cdc2 but not the fly CDK2 homolog, cdc2c. Given that p16- and p21-like CDK inhibitors have not been found for cdc2, it is possible that cdc2 and the rest of the CDKs are negatively regulated by different families of proteins. Alternatively, the activity of each CDK could be modulated by both types of negative regulators. In both flies and mammals, cdc2/cyclin A is inactivated during early mitosis by degradation of cyclin A, while degradation of cyclin B

occurs later at the metaphase/anaphase transition (Minshull et al., 1990, EMBO J. 9:2865-2875; Whitfield et al., 1990, EMBO J. 9:2563-2572). The mechanism of such differential inactivation of cdc2/cyclin is unknown. Our data indicate that lats specifically modulates cdc2/cyclin A activity but not cdc2/cyclin B activity: cyclin A but not cyclin B mutants interact with lats genetically; *lats* mutant cells abnormally accumulate cyclin A but not cyclin B (Figures 5A-N).

Finally, overexpression of cdc2 and cyclin A has been reported in multiple types of human tumors (Wang et al., 1990, Nature 343:555-557; Keyomarsi and Pardee, 1993, Proc. Natl. Acad. Sci. USA 90:1112-1116; Arany et al., 1994, Surg. Onc. 3:153-159). Negative regulators of CDK/Cyclins (*e.g.*, p16) have been shown to function as tumor suppressors in mammals (Serrano et al., 1996, Cell 85:27-37). The biochemical and genetic data for lats provided herein suggest that lats would behave as a tumor suppressor in mammals.

<u>EXAMPLE 2: Mice Deficient for Lats Develop Soft Tissue Sarcomas, Ovarian Tumors and Pituitary Dysfunction</u>

Materials and Methods

Generation of *lats*$^{-/-}$ mice

Mouse *lats* genomic DNA was isolated by screening a 129 library (Stratagene) using a mouse *lats* cDNA as a probe. A SalI fragment from the cDNA was subcloned into a pBS vector. We cleaved this construct at the EcoRV site (Figure 6B), and inserted a 1.8 kb fragment encoding PGK-neo. We subsequently digested with BamHI and XhoI and inserted a 3 kb PGK-TK gene cassette.

D3 embryonic stem (ES) cells were electroporated with the SfiI linearized vector, and selected in 0.3 mg/ml G418 and 2 μM ganciclovir media for incorporation of the vector. The ES cell clones analyzed underwent homologous recombination at the lats locus (Figure 6B). For genotyping, genomic DNA from the ES cells was digested with BamHI and EcoRV and analyzed by Southern blotting using the BamHI-EcoRI probe from the vector (Figure 6C). The double digest of the wild type allele generates a 3.5 kb fragment that hybridizes to the probe, while double digest of the disrupted allele generates a 5.8 kb fragment that hybridizes to the probe. *Lats* heterozygous ES cells were microinjected into CS7BL/6 blastocysts which were transplanted into uteri of pseudopregnant ICR mice. Chimeric male progeny were crossed to CS7BL/6 females. Germline transmission of the disrupted allele was detected in agouti progeny by Southern blotting.

Cell culture and protein analysis

Proteins were extracted from whole-cell lysates of *lats*$^{-/-}$ mouse embryonic fibroblasts (MEFs) derived from 13-days post-coitum mouse embryos, separated using SDS-PAGE, transferred and probed with rabbit polyclonal anti-lats antibody, followed by enhanced chemiluminescence detection (Amersham).

Histopathological examinations

For morphological evaluation, tissues were fixed in 10% neutral buffered formalin at 4°C overnight, dehydrated with ethanol, embedded in paraffin, and sectioned into 4 to 5 mm sections. Paraffin sections were prepared by standard procedures and stained with hematoxylin and eosin.

Gonadotropin treatment

Mice were injected intraperitoneally with FSH administered in the form of 5 IU of pregnant mare serum gonadotropin (Sigma). 44-46 hours later, mice were injected intraperitoneally with LH in the form of 5 IU of human chorionic gonadotropin (Sigma).

Pituitary hormone measurements

We used 20 *lats*$^{-/-}$ and 20 *lats*$^{+/-}$ age, sex, and estrus cycle matched females and males for these analyses. Mouse serum levels of PRL, LH, GH, FSH, and TSH were determined in pooled serum samples by double antibody radioimmunoassays (RIAs). These sensitive, specific mouse pituitary hormone RIAs were developed by A. F. Parlow, and are distributed to the scientific research community via the National Hormone & Pituitary Program of NIDDK, NIH (see http://www.humc.edu/hormones).

UVB and DMBA tumorigenic treatments

UVB and DMBA treatments were performed as described by Serrano et al. (1996, Cell 85:27-37). Briefly, skin tumors were induced by first applying a single dose of 9,10-dimethyl-1,2-benzathralene (DMBA; 50 µl of an 0.5% solution in acetone) to the dorsal surface of the mouse 1 to 5 days after birth. This treatment was followed by exposure to ultraviolet B (UVB) irradiation approximately three times per week for, on average, 27 treatments, with an initial exposure of approximately 100 mJ/cm2, increasing the dosage by 10% per treatment to a maximum of 700 mJ/cm2.

Results and Discussion

Targeted Disruption of the mouse *lats* gene

A 17.5 kilobase *lats* genomic clone obtained from a mouse 1295V library was used to construct a targeting vector for homologous recombination by positive-negative selection (Mansour et al., 1988, Nature 336:348-352; Capecchi, 1989, Science 244:1288-1292) as shown in Figure 6B. A PGK-neo cassette was inserted in inverse orientation into an exon of the lats clone resulting in the removal of amino acid sequence corresponding to amino acids 756-1130 of human lats (Figure 6A). We electroporated D3 embryonic stem cells (Gossler et al., 1986, Proc. Natl. Acad. Sci USA 83:9065-9069) with the lats-neo construct; single clones resistant to G418 and ganciclovir were screened by Southern blot hybridization using a mouse lats probe 5' to the portion of the *lats* gene contained in the targeting vector (Figure 6B). Restriction enzyme digestion of the wild-type lats locus with

BamHI and EcoRV generated a 3.5 kb fragment, while the correctly targeted, disrupted locus generated a 5.8 kb fragment (Figures 6B and C). A targeting frequency of approximately 1 in 100 was observed. Male chimeras transmitted the targeted *lats* allele through the germline, as demonstrated by Southern blot analysis of tail DNA. Immunoprecipitation and western blotting of *lats*$^{-/-}$ mouse embryonic fibroblast lysates (derived from 13.5 days post-coitum embryos (dpc)) with polyclonal anti-human lats antibody confirmed the absence of lats protein in homozygous null embryos (Figure 6D).

Growth and viability of *lats*$^{-/-}$ mice

When assessed at 3 weeks of age, the number of *lats* mutant animals was drastically lower than expected; 8% of pups genotyped from double heterozygote matings were *lats*$^{-/-}$, significantly lower than the expected frequency of 25%. Just before birth at embryonic day 18.5 (dpc), however, live homozygous embryos were found at the frequency predicted by Mendelian law (25%). The majority of homozygotes died within the first day of life, and their death was associated with internal hemorrhage into vital organs. The reason that some *lats*$^{-/-}$ mice survived while most did not is uncertain, but the mixed genetic background (strains 129 and C57BL/6) of the mice could be contributory. The weight of homozygous embryos at birth was approximately 70% of that of wild-type embryos (*lats*$^{+/+}$, 1.5 ± 0.3 g; *lats*$^{-/-}$, 1.3 ± 0.3 g; *lats*$^{-/-}$, 1.1 ± 0.1 g). All of these *lats*$^{-/-}$ mice were growth retarded. Most of the surviving *lats* homozygous mutant animals gained weight slowly, attaining only 60-70% of normal weight as adults (Figures 7A and B). A representative growth curve for lats deficient mice is shown in Figure 7B. To determine if there was a correlation between weight and growth, skeletal growth and organ weight was examined. By radiography, we observed differences in the skull and longitudinal bones that corresponded to the decrease in the size of the mouse. The decreased body weight was not due to the animals being leaner than their wild-type siblings, because the weight of most organs tested had decreased in proportion to the whole body weight. Exceptions were seen in a few particularly reduced organs, including the testis and the ovary.

Pituitary dysfunction

Male *lats*$^{-/-}$ mice displayed decreased fertility although histopathological examination of the testis did not reveal obvious structural abnormalities. *Lats*$^{-/-}$ females all displayed severe fertility defects, and approximately 60% of the females were completely sterile. Ovaries from all lats deficient females examined contained far fewer follicles than age and parity matched ovaries from *lats*$^{+/+}$ females (Figures 8A-D). The majority of follicles observed were primary and secondary follicles. Formation of the antrum was much less prominent than in normal mice. The follicles also contained fewer degenerative granulosa cells, which are common in atretic follicles in normal mice. Moreover, the formation of a corpus luteum was not detected (Figures 8A-D). These histological findings suggest an impairment of ovulation in *lats*$^{-/-}$ ovaries, and potential endocrine dysfunction.

The amount of breast epithelial tissue was markedly decreased in *lats*$^{-/-}$ females, with some females displaying a complete lack of macroscopic nipple-formation (Figures 9D and E). Histologically, the mammary glands of *lats*$^{-/-}$ mice were frequently reduced to a "fat pad" devoid of a ductular component (Figure 9F). This too suggested an endocrine component to the phenotype, as loss of lats may alter the levels of hormones that affect breast development.

Estrus is another indicator of endocrine function. Vaginal smears taken from control ($^{+/+}$) mice showed that they cycled through proestrus, estrus, metestrus, and diestrus in 4 days as described previously for normal mice (Nelson et al., 1982, Biol. Reprod. 27:327-339). In contrast, infertile *lats*$^{-/-}$ females did not cycle, and remained in continuous metestrus, an observation that further characterizes their infertility. The abnormal estrus cycle might reflect an underlying problem in signaling between the pituitary and the ovary. To determine if *lats*$^{-/-}$ ovaries could respond to appropriate gonadotropin stimulation, young adult mice (7 *lats*$^{-/-}$ females; 2 control females) were injected with a Follicle-Stimulating Hormone (FSH) analog (pregnant mare serum), and 46 hours later with a Luteinizing Hormone (LH) preparation (human chorionic gonadotropin). This treatment allowed *lats*$^{-/-}$ females to cycle into a prolonged period of estrus, confirming that the temporal synchronization of the levels of endogenous FSH and/or LH in *lats*$^{-/-}$ females is deficient.

In all *lats* knock-out mouse pituitaries examined (n=20), hyperplastic changes were readily apparent. There were multiple foci of atypical cells showing irregularly shaped nuclei with an increased content of chromatin and variability in size (Figures 10A and B). This histological atypia was accompanied by a pathological dysfunction of the pituitary. For example, hormone measurements in sera from *lats*$^{-/-}$ females revealed: the levels of LH were 3-fold lower than controls (Figure 10C); the PRL levels were less than half of controls (Figure 10D); and Growth Hormone (GH) levels were reduced by 25%. These deficiencies may have resulted from an unbalanced increase in the number of certain types of cells in this normally highly organized tissue. The reduced serum GH level may contribute to the reduced size of *lats*$^{-/-}$ mice. The diminished levels of serum LH could account for the lack of proper follicular maturation and differentiation, as well as the infertility observed in female *lats*$^{-/-}$ animals, with greater atypia in the pituitary leading to the more severe phenotype. The PRL and LH defects, together, account for both the lack of mammary gland development and the corpus luteum insufficiency syndrome which these animals display.

Interestingly, serum levels of pituitary Follicle Stimulating Hormone (FSH) (Figure 10E) and Thyroid Stimulating Hormone (TSH) are normal. The observation that *lats*$^{-/-}$ mice display a selective LH deficiency while sustaining normal FSH production supports a model of differential regulation of the two gonadotropins. This is in accordance with data from Lee and coworkers demonstrating that the transcription factor NGFI-A specifically regulates LH-ß (Lee et al., 1996, Science 273:1219-1221). *Lats*$^{-/-}$ mice thereby provide a model for the human reproductive dysfunction of isolated LH hypogonadotropic hypogonadism.

It is interesting to note that the pituitary deficiencies of *lats*$^{-/-}$ mice resembles those of other cell cycle regulator knock-out mice, such as the *Rb*$^{+/-}$, *p53*$^{-/-}$, and *p27*$^{-/-}$ mice. In these examples, pituitary cells and other endocrine organs appear to be crucially dependent on cell cycle regulation for their proper development. Indeed, tumor suppressors may play such a key role in the pituitary because critical function in this tissue allows for a link between control of single cell proliferation and total organismal growth and survival.

Tumor development in *lats*$^{-/-}$ mice

Another cause for the infertility of lats-deficient females is that they developed ovarian stromal cell tumors by the age of 3 months. The body of the normal ovary consists of spindle-shaped cells, reticular fibers and ground substance which together constitute the ovarian stroma, in which numerous follicles are embedded (Wheater et al., 1987, Functional Histology; Figure 8A). Stromal cell tumors in *lats*$^{-/-}$ mice obliterated the normal structure of the ovary, eliminating follicles progressively (Figures 8B and D). These stromal cell tumors are probably not resultant from pituitary dysfunction, as stromal cell tumors are most often local events (Clement, "Histology of the Ovary" in Histology For Pathologists, Second Ed., Sternberg, ed. (Lippincott-Raven, 1997) pp 934-935). Some *lats*$^{-/-}$ females were able to give birth to one litter, then became infertile as the stromal cell tumors expanded into the remaining functional ovary. To date, these stromal cell tumors have not yet displayed signs of malignancy. Stromal cell tumors were observed in all lats deficient mice examined (n=22), and extended throughout the entire ovary as determined by serial sectioning.

Additional evidence supporting the role of lats in mammalian tumorigenesis is that thus far, approximately 15% of all *lats*$^{-/-}$ females (n=28) between 4 to 10 months of age have developed large soft tissue sarcomas with metastases to vital organs (*e.g.* the lungs) (Figures 11A-C). Taking genetic backgrounds into consideration and using litter mates as controls, approximately 57% of all *lats*$^{-/-}$ animals developed soft tissue sarcomas. Histology revealed that these sarcomas consist of pleiomorphic, spindle-shaped cells with mitotic figures (Figure 11C), and they displayed immunohistochemical features of fibrosarcomas. No tumors were detected in control mice (n=80). It is possible that the spontaneous rate of tumor formation is even higher in *lats*$^{-/-}$ mice as some tumors may be occult, and therefore not readily identifiable. *Lats*$^{+/-}$ mice remained tumor free up to 8 months of age. This phenotype is consistent with that observed in heterozygotes for the CDK inhibitors, p27 and p16 (Fero et al., 1996, Cell 85:733-744; Kiyokawa et al., 1996, Cell 85:721-732; Nakayama et al., 1996, Cell 85:707-720; and Serrano et al., 1996, Cell 85:27-37).

The susceptibility of *lats*$^{-/-}$ mice to tumor induction by carcinogens was assessed using a standard two-stage protocol consisting of a single application of 9,10-dimethyl-1,2-benzanthracene (DMBA) followed by repeated exposure to ultraviolet B rays (Serrano et al., 1996, Cell 85:27-37). By 7 weeks of age, over 71% of *lats*$^{-/-}$ animals developed soft tissue sarcomas, whereas all of the control animals remained free of obvious tumors. The

frequency of tumor development in these *lats*$^{-/-}$ animals is particularly impressive, given the fact that C57BL/6 mice are poorly susceptible to the development of skin tumors (as reviewed by DiGiovanni, 1991, Pharm. & Ther. 54:63-128). The spontaneous and induced tumors observed in *lats*$^{-/-}$ animals provide clear evidence of the role of lats in mammalian tumorigenesis, and attest to the functional conservation of lats.

Although both *Drosophila* and mice develop tumors when lats is inactivated, the correlation between genotype and phenotype differs between the two organisms. While every *lats*$^{-/-}$ cell overproliferates in mosaic flies (Xu et al. 1995, Development 121:1053-1063), only certain tissues in *lats*$^{-/-}$ mice develop tumors. This phenotypic difference could be due to the increased complexity in cell cycle control in mammals. For example, in *Drosophila*, cdc2/Cyclin A functions at both the G1/S and G2/M transitions in the cell cycle (Whitfield et al., 1990, EMBO J. 9:2563-2573; Knoblich and Lehner, 1993, EMBO J. 12:65-74; Knoblich et al., 1994, Cell 77:117-120; Sauer et al., 1995, Genes & Devel. 9:1237-1239; Sigrist and Lehner, 1997, Cell 90:671-681; Sprenger et al., 1997, Curr. Biol. 7:488-499; Thomas et al., 1997, Genes & Dev. 11:1289-1298). In mammals, however, a different CDK, CDK2, complexes with cyclin A to regulate the G1/S transition, while cdc2/cyclin A functions in the G2/M transition (Girard et al., 1991, Cell 67:1169-1179; Tsai et al., 1991, Nature 353:174-177; Hamaguchi et al., 1992, J. Cell. Biol. 117:1041-1053; Pagano et al., 1992, EMBO J. 11:961-967; Rosenblatt et al., 1992, Proc. Natl. Acad. Sci. USA 89:2824-2828; Hunter and Pines, 1994, Cell 79:573-582; Resnitzky et al., 1995, Mol. Cell Biol. 15:4347-4352). We have shown that lats negatively regulates cdc2/cyclin A, but does not appear to interact with CDK2. Thus, while inactivation of *lats* in *Drosophila* affects regulation of both the G1/S and G2/M transitions of the cell cycle, the removal of lats function in mammalian cells only affects control of the G2/M transition. This result is consistent with the fact that human cancers are often caused by mutations in multiple, non-homologous cell cycle control pathways (Kinzler and Vogelstein, 1996, Cell 87:159-170; Weinberg, 1996, Sci. Am. 275:62-70). The higher degree of redundancy in mammalian genomes could also contribute to the phenotypic difference between fly and mouse *lats* mutants.

Although most human tumor suppressors that have been characterized function in the regulation of G1/S (Brown, 1997, Adv. Genet. 36:45-135), there are indications that deregulation of G2/M and M controls may also contribute to tumorigenesis in mammals. For example, p53 is involved in the regulation of both G1/S and G2/M (and M) (Cross et al., 1995, Science 267:1353-1356; Hermeking et al., 1997, Mol. Cell 1:3-11). Inactivation of p53 in mice leads to impressive tumor development, while the disruption of p21, the p53-downstream effector for G1/S, has no tumorigenic effect (Deng et al., 1995, Cell 82:675-684). Recent data also suggests that Rb plays a role in G2/M in addition to its G1/S involvement (Niculescu et al., 1998, Mol. Cell Biol. 18:629-643). Furthermore, overexpression of cdc2 and mitotic cyclins has been reported in multiple types of human tumors (Arany et al., 1994, Surg, Oncol. 3:153-159; Keyomarsi and Pardee, 1993, Proc.

Natl. Acad. Sci USA 90:1112-1116; Wang et al., 1990, Nature 343:555-557). Signaling through the RHAMM receptor affects cell proliferation by down-regulation of cdc2 and cyclin B transcripts and proteins and results in reversal of tumorigenesis (Mohapatra et al., 1996, J. Exper. Med. 183:1663-1668). The tumorigenic phenotype of *lats*$^{-/-}$ mice further supports the notion that the regulation of G2/M or M also plays a role in mammalian tumor development.

A comparison between lats- and p16-knock-out mice is interesting in several respects. Both lats and p16 are negative regulators of CDKs. In addition, lats-knock-out mice resemble p16-knock-out animals (Serrano et al., 1996, Cell 85:27-37) in that homozygotes develop tumors at an early age while heterozygotes do not. Although different types of tumors are observed in these two mutants (*e.g.*, ovarian tumors in *lats*$^{-/-}$ mice and lymphomas in *p16*$^{-/-}$ mice), both types of knock-out mice develop soft tissue sarcomas. The frequencies of spontaneous soft tissue sarcomas between these two knock-out mutants cannot be directly compared due to protocol differences, however, induced tumors in these animals were obtained using the same induction protocol (Serrano et al., 1996, Cell 85:27-37). Interestingly, the frequency of induced tumor formation in *lats*-knock-out mice is even higher than that observed for *p16*$^{-/-}$ mice. Over 71% of *lats*$^{-/-}$ animals developed soft tissue sarcomas by 7 weeks of age. Only 10% of the *p16*$^{-/-}$ mice developed tumors by 7 weeks of age, and 60% of them displayed tumors between the ages of 6-12 weeks (Serrano et al., 1996, Cell 85:27-37).

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. A recombinant non-human animal in which a *lats* gene has been inactivated by a method comprising introducing a nucleic acid into the animal, or an ancestor thereof, which nucleic acid comprises a non-*lats* sequence flanked by *lats* genomic sequences that promote homologous recombination, such that said non-*lats* sequence replaces the nucleotide sequence encoding the Lats C-terminal domain 1, the Lats C-terminal domain 2, the Lats C-terminal domain 3, and a portion of the kinase domain of the lats protein encoded by the *lats* gene.

2. The recombinant non-human animal of claim 1 in which the non-*lats* sequence replaces the nucleotide sequence encoding the amino acids that correspond to amino acids 756-1130 of human lats, as depicted in Figure 12 (SEQ ID NO:2).

3. The recombinant non-human animal of claim 1 which is a mouse.

4. The recombinant non-human animal of claim 3 in which the *lats* gene contains a lats coding sequence of SEQ ID NO:3.

5. The recombinant non-human animal of claim 1 in which both alleles of the *lats* gene have been inactivated.

6. A method for screening a potential therapeutic compound for activity in treating or preventing cancer comprising administering the compound to the recombinant non-human animal of claim 1; and comparing the size or progression of the cancer in the recombinant non-human animal to which the compound was administered with the size or progression of the cancer in the same recombinant non-human animal prior to administration of the compound or in a recombinant non-human animal that was not so administered or to a standard size or progression of the cancer for such same or a recombinant non-human animal that was not so administered, wherein a decrease in the size or progression of the cancer in the recombinant non-human animal administered the compound as compared to the same animal prior to the administration or to the recombinant non-human animal not so administered or to the standard size or progression of the cancer, indicates that the compound has activity in treating or preventing cancer.

7. The method of claim 6 in which the non-*lats* sequence replaces the nucleotide sequence encoding the amino acids that correspond to amino acids 756-1130 of human lats, as depicted in Figure 12 (SEQ ID NO:2).

8. The method of claim 6 in which the recombinant non-human animal is a mouse.

9. The method of claim 8 in which the *lats* gene contains the lats coding sequence of SEQ ID NO:3.

10. The method of claim 6 in which both alleles of the *lats* gene have been inactivated.

11. The method of claim 6 in which the compound is screened for activity in treating or preventing soft tissue sarcomas.

12. The method of claim 6 in which the compound is screened for activity in treating or preventing ovarian tumors.

13. A method for screening a potential therapeutic compound for activity in treating or preventing cancer comprising recombinantly expressing the compound in the recombinant non-human animal of claim 1; and comparing the size or progression of the cancer in the recombinant non-human animal in which the compound was expressed with the size or progression of the cancer in the same recombinant non-human animal prior to expression of the compound or in a recombinant non-human animal in which the compound was not so expressed or to a standard size or progression of the cancer for such same or a recombinant non-human animal in which the compound was not so expressed, wherein a decrease in the size or progression of the cancer in the recombinant non-human animal in which the compound was expressed as compared to the same animal prior to the expression of the compound or to the recombinant non-human animal in which said compound was not so expressed or to the standard size or progression of the cancer, indicates that the compound has activity in treating or preventing cancer.

14. A method for screening a potential therapeutic compound for activity in treating or preventing skin cancer comprising administering the compound to a *lats* knock-out animal having skin tumors induced by exposure to at least one carcinogen; and comparing the size or progression of the skin tumors on the *lats* knock-out animal to which the compound was administered with the size or progression of skin cancers on the same *lats* knock-out animal prior to administration of the compound or on a *lats* knock-out animal in which skin tumors have also been induced by exposure to said at least one carcinogen but which has not been administered the compound or to a standard size or progression of the skin tumors for such same or a *lats* knock-out animal that was not so administered, wherein a reduction in the size or progression of the skin tumors in the *lats* knock-out animal administered the compound as compared to the same animal prior to

administration of the compound or to the animal not so administered or to the standard size or progression of the skin tumors, indicates that the compound has activity in treating or preventing skin cancer.

15. The method of claim 14 in which the *lats* knock-out animal has at least one *lats* gene which was inactivated by promoting homologous recombination between *lats* genomic sequences and a nucleic acid having non-*lats* sequences flanked by genomic sequences.

16. The method of claim 15 in which the non-*lats* sequence replaces the nucleotide sequence encoding the Lats C-terminal domain 1, the Lats C-terminal domain 2, the Lats C-terminal domain 3, and a portion of the kinase domain of the lats protein encoded by the *lats* gene.

17. The method of claim 15 in which the non-*lats* sequence replaces the nucleotide sequencel encoding the amino acids that correspond to amino acids 756-1130 of human lats, as depicted in Figure 12 (SEQ ID NO:2).

18. The method of claim 14 in which the *lats* knock-out animal is a mouse.

19. The method of claim 18 in which the *lats* gene contains the lats coding sequence of SEQ ID NO:3.

20. The method of claim 14 in which both alleles of the *lats* gene have been inactivated.

21. The method of claim 14 in which the skin tumors were induced by 9,10-dimethyl-1,2-benzanthracene and repeated exposure to ultraviolet B radiation.

22. The method of claim 14 in which the potential therapeutic compound is administered topically.

23. A method for screening a potential therapeutic compound for activity in treating or preventing skin cancer comprising recombinantly expressing the compound in a *lats* knock-out animal having skin tumors induced by exposure to at least one carcinogen; and comparing the size or progression of the skin tumors on the *lats* knock-out animal in which the compound was expressed with the size or progression of skin cancers on the same *lats* knock-out animal prior to expression of the compound or on a *lats* knock-out animal in which skin tumors have also been induced by exposure to said at least one carcinogen but in which the compound has not been expressed or to a standard size or progression of the skin

tumors for such same or a *lats* knock-out animal in which the compound was not so expressed, wherein a reduction in the size or progression of the skin tumors in the *lats* knock-out animal in which the compound was expressed as compared to the same animal prior to expression of the compound or to the animal in which the compound was not so expressed or to the standard size or progression of the skin tumors, indicates that the compound has activity in treating or preventing skin cancer.

24. A method for screening a potential therapeutic compound for activity in treating or preventing a disease or disorder associated with pituitary dysfunction comprising administering the compound to a *lats* knock-out animal; and comparing the level of an indicator of pituitary dysfunction in the *lats* knock-out animal to which the compound has been administered to the level of the indicator in the same *lats* knock-out animal prior to administration of the compound or to a *lats* knock-out animal that has not been administered the compound or to a standard level of the indicator for such same or a *lats* knock-out animal that was not so administered, wherein a change in the indicator toward the level of the indicator in a wild type animal not afflicted with a pituitary dysfunction as compared to the same animal prior to administration of the compound or to the animal not so administered or to the standard level of the indicator, indicates that the compound is active to treat or prevent a disease or disorder associated with pituitary dysfunction.

25. The method of claim 24 in which the *lats* knock-out animal has at least one *lats* gene which was inactivated by promoting homologous recombination between *lats* genomic sequences and a nucleic acid having non-*lats* sequences flanked by genomic sequences.

26. The method of claim 25 in which the non-lats sequence replaces the nucleotide sequence encoding the Lats C-terminal domain 1, the Lats C-terminal domain 2, the Lats C-terminal domain 3, and a portion of the kinase domain of the lats protein encoded by the *lats* gene.

27. The method of claim 25 in which the non-*lats* sequence replaces the nucleotide sequence encoding the amino acids that correspond to amino acids 756-1130 of human lats, as depicted in Figure 12 (SEQ ID NO:2).

28. The method of claim 24 in which the *lats* knock-out animal is a mouse.

29. The method of claim 28 in which the *lats* gene contains a lats coding sequence of SEQ ID NO:3.

30. The method of claim 24 in which both alleles of the *lats* gene have been inactivated.

31. The method of claim 24 in which the indicator is fertility.

32. The method of claim 24 in which the indicator is ovulation.

33. The method of claim 24 in which the indicator is linear growth.

34. The method of claim 24 in which the indicator is serum levels of luteinizing hormone, growth hormone or prolactin.

35. The method of claim 24 in which the disease or disorder is LH hypogonadotropic hypogonadism.

36. A method for screening a potential therapeutic compound for activity in treating or preventing a disease or disorder associated with pituitary dysfunction comprising recombinantly expressing the compound in a *lats* knock-out animal; and comparing the level of an indicator of pituitary dysfunction in the *lats* knock-out animal in which the compound has been expressed to the level of the indicator either in the same *lats* knock-out animal prior to expression of the compound or to a *lats* knock-out animal in which the compound has not been expressed or to a standard level of the indicator for such same or a *lats* knock-out animal in which the compound was not so expressed, wherein a change in the indicator toward the level of the indicator in a wild type animal not afflicted with a pituitary dysfunction as compared to the same animal prior to expression of the compound or to the animal in which the compound was not so expressed or to the standard level of the indicator, indicates that the compound is active to treat or prevent a disease or disorder associated with pituitary dysfunction.

37. The method of claim 6, 14 or 24 in which the compound is purified.

38. A method for treating a cancer that has been shown to be refractory to a chemotherapy or radiation therapy in a subject in need of such treatment comprising administering to the subject a therapeutically effective amount of a molecule that promotes lats function.

39. The method of claim 38 in which the subject is a human.

40. The method of claim 38 in which the molecule is a lats protein.

41. The method of claim 38 in which the molecule is a protein having an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8.

42. The method of claim 38 in which the molecule is a protein having the amino acid sequence of SEQ ID NO:2.

43. The method of claim 38 in which the molecule is a lats analog or derivative that has activity to promote lats function.

44. The method of claim 38 in which the molecule is a protein encoded by a first nucleic acid that is hybridizable under conditions of low stringency to a second nucleic acid having a nucleotide sequence that is the reverse complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, and SEQ ID NO:7, said protein having activity to inhibit cell overproliferation.

45. The method of claim 38 in which the molecule is a protein consisting of at least 20 contiguous amino acids of a protein having an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8, said protein having activity to inhibit cell overproliferation.

46. The method of claim 38 in which the molecule is a protein comprising a domain of a lats protein selected from the group consisting of a lats C-terminal domain 3 (LCD3), lats C-terminal domain 2 (LCD2), lats C-terminal domain 1 (LCD1), kinase domain, lats flanking domain (LFD), lats split domain 1 (LSD1), lats split domain 2 (LSD2), and SH3-binding domain, said protein having activity to inhibit cell overproliferation.

47. The method of claim 40 in which the lats protein is phosphorylated.

48. The method of claim 47 in which the lats protein is phosphorylated on a serine or threonine residue within 20 residues upstream of the amino acid sequence Ala-Pro-Glu in a subdomain eight of a kinase domain of said lats protein.

49. The method of claim 48 in which the lats protein is phosphorylated at a serine residue corresponding to serine 909 of the human lats amino acid sequence, as depicted in Figure 12 (SEQ ID NO:2).

50. The method of claim 43 in which the lats analog or derivative has a threonine or serine residue within 20 residues upstream of the amino acid sequence Ala-Pro-Glu in a

subdomain eight of a kinase domain of said lats analog or derivative substituted with an aspartate or glutamate residue.

51. The method of claim 50 in which the lats analog or derivative has a glutamate residue substituted for a serine residue at the residue corresponding to serine 909 of the human lats amino acid sequence, as depicted in Figure 12 (SEQ ID NO:2).

52. The method of claim 38 in which said molecule is a chimeric protein comprising a fragment of a lats protein, said fragment consisting of at least 20 contiguous amino acids of said lats protein, fused via a covalent bond to an amino acid sequence of a second protein, said second protein not being a lats protein, said chimeric protein having activity to inhibit cell overproliferation.

53. The method of claim 38 in which said cancer has been shown to be refractory to radiation therapy.

54. The method of claim 38 in which said cancer has been shown to be refractory to chemotherapy.

55. The method of claim 54 in which said chemotherapy kills cancer cells during S phase of the cell cycle.

56. The method of claim 54 in which said chemotherapy kills cancer cells during mitosis.

57. The method of claim 38 which further comprises administering one or more chemotherapeutic agents to the subject.

58. The method of claim 57 in which said one or more chemotherapeutic agents are administered concurrently with the administration of said molecule.

59. The method of claim 57 in which said one or more chemotherapeutic agents are administered subsequent to the administration of said molecule.

60. The method of claim 38 in which said molecule is a nucleic acid comprising a nucleotide sequence encoding a lats protein.

61. The method of claim 60 in which said nucleotide sequence is SEQ ID NO:1.

62. The method of claim 60 in which said nucleotide sequence encodes a protein having the amino acid sequence of SEQ ID NO:2.

63. The method of claim 38 in which said molecule is a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid that is the reverse complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, and SEQ ID NO:7.

64. A method for treating a cancer that has been shown to be refractory to a chemotherapy or radiation therapy in a subject in need of such treatment comprising administering to the subject a therapeutically effective amount of a cell that expresses a recombinant nucleic acid that promotes lats function.

65. The method of claim 64 in which said nucleic acid comprises the nucleotide sequence of SEQ ID NO:1.

66. The method of claim 64 in which said nucleic acid comprises a nucleotide sequence encoding a protein having the amino acid sequence of SEQ ID NO:2.

67. A kit comprising in one or more containers a therapeutically effective amount of a molecule selected from the group consisting of a lats protein, a lats derivative, a lats analog, a nucleic acid encoding a lats protein, a nucleic acid encoding a lats derivative, and a nucleic acid encoding a lats analog; and at least one chemotherapeutic agent.

68. A purified complex of a lats protein and a cdc2 protein.

69. The purified complex of claim 68 in which the proteins are human proteins.

70. The purified complex of claim 68 in which the lats protein is phosphorylated.

71. The purified complex of claim 70 in which the lats protein is phosphorylated on a serine or threonine residue within 20 residues upstream of the amino acid sequence Ala-Pro-Glu in a subdomain eight of a kinase domain of said lats protein.

72. The purified complex of claim 71 in which the lats protein is phosphorylated at a serine residue corresponding to serine 909 of the human lats amino acid sequence, as depicted in Figure 12 (SEQ ID NO:2).

73. A purified complex selected from the group consisting of a complex of a derivative of a lats and a cdc2 protein, a complex of a lats protein and a derivative of a cdc2,

and a complex of a derivative of a lats protein and a derivative of a cdc2 protein, in which the derivative of the lats protein is able to form a complex with a wild-type cdc2 protein and the derivative of the cdc2 is able to form a complex with a wild-type lats protein.

74. The purified complex of claim 73 in which the derivative of the lats protein and/or the cdc2 protein is fluorescently labeled.

75. The purified complex of claim 73 in which the lats derivative has a threonine or serine residue within 20 residues upstream of the amino acid sequence Ala-Pro-Glu in a subdomain eight of a kinase domain of said lats derivative substituted with an aspartate or glutamate residue.

76. The purified complex of claim 75 in which the lats derivative has a glutamate residue substituted for a serine residue at the residue corresponding to serine 909 of the human lats amino acid sequence, as depicted in Figure 12 (SEQ ID NO:2).

77. The purified complex of claim 73 in which the lats derivative is a fragment of a lats protein consisting of the amino acid sequence corresponding to amino acids 15-585 of the amino acid sequence of human lats, as depicted in Figure 12 (SEQ ID NO:2).

78. A chimeric protein comprising a fragment of a lats protein consisting of at least 6 amino acids fused via a covalent bond to a fragment of a cdc2 protein consisting of at least 6 amino acids.

79. The chimeric protein of claim 78 in which the fragment of the lats protein is a fragment capable of binding the cdc2 protein and in which the fragment of the cdc2 protein is a fragment capable of binding the lats protein.

80. The chimeric protein of claim 78 in which the fragment of the lats protein has an amino acid sequence corresponding to amino acids 15 to 585 of the amino acid sequence of human lats, as depicted in Figure 12 (SEQ ID NO:2).

81. The chimeric protein of claim 79 in which the fragment of the lats protein and the fragment of the cdc2 protein form a lats-cdc2 complex.

82. An antibody which immunospecifically binds the complex of claim 68 or a fragment or derivative of said antibody containing the binding domain thereof.

83. The antibody of claim 82 which does not immunospecifically bind a lats protein or a cdc2 protein that are not part of a lats-cdc2 complex.

84. An isolated nucleic acid or an isolated combination of nucleic acids comprising a nucleotide sequence encoding a lats protein and a nucleotide sequence encoding a cdc2 protein.

85. The isolated nucleic acid or isolated combination of nucleic acids of claim 84 which are nucleic acid vectors.

86. An isolated nucleic acid that comprises a nucleotide sequence encoding the chimeric protein of claim 78.

87. A cell containing the nucleic acid of claim 84, which nucleic acid is recombinant.

88. A cell containing the nucleic acid of claim 86, which nucleic acid is recombinant.

89. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of the complex of claim 68; and a pharmaceutically acceptable carrier.

90. The pharmaceutical composition of claim 89 in which the proteins are human proteins.

91. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of the complex of claim 73; and a pharmaceutically acceptable carrier.

92. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of the chimeric protein of claim 79; and a pharmaceutically acceptable carrier.

93. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of the antibody of claim 83 or a fragment or derivative of said antibody containing the binding domain thereof; and a pharmaceutically acceptable carrier.

94. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of the nucleic acids or combination of nucleic acids of claim 84; and a pharmaceutically acceptable carrier.

95. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of the isolated nucleic acid of claim 86; and a pharmaceutically acceptable carrier.

96. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of the recombinant cell of claim 87; and a pharmaceutically acceptable carrier.

97. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of the recombinant cell of claim 88; and a pharmaceutically acceptable carrier.

98. A method of producing a complex of a lats protein and a cdc2 protein comprising growing a recombinant cell containing the nucleic acid of claim 84 such that the encoded lats and cdc2 proteins are expressed and bind to each other, and recovering the expressed complex of the lats protein and the cdc2 protein.

99. A method of diagnosing or screening for the presence of or a predisposition for developing a disease or disorder characterized by an aberrant level of a complex of a lats protein and a cdc2 protein in a subject comprising measuring the level of said complex, RNA encoding the lats and the cdc2 proteins, or functional activity of said complex, in a sample derived from the subject, in which an increase or decrease in the level of said complex, said RNA encoding lats and cdc2, or functional activity of said complex in the sample, relative to the level of said complex, said RNA encoding lats and cdc2, or functional activity of said complex found in an analogous sample from a subject not having the disease or disorder or a predisposition for developing the disease or disorder, indicates the presence of the disease or disorder or a predisposition for developing the disease or disorder.

100. A kit comprising in one or more containers a substance selected from the group consisting of a complex of a lats and a cdc2 protein, an antibody against said complex, nucleic acid probes capable of hybridizing to RNA of lats and RNA of cdc2, or pairs of nucleic acid primers capable of priming amplification of at least a portion of a gene for lats and a gene for cdc2.

101. A method for modulating the activity of cdc2 comprising administering a molecule that promotes, inhibits, or antagonizes lats function.

102. A method for inhibiting the activity of cdc2 comprising administrating a molecule that promotes lats function.

103. A method for increasing the activity of cdc2 comprising administering a molecule that inhibits or antagonizes lats function.

104. A method for treating or preventing a disease or disorder associated with an aberrantly high level of cdc2 in a subject in need of such treatment or prevention comprising administering to the subject a therapeutically effective amount of a molecule that promotes lats function.

105. The method of claim 104 in which said molecule is selected from the group consisting of a lats protein, a lats derivative or analog that promotes lats function, a nucleic acid encoding a lats protein, and nucleic acid encoding a lats derivative or analog that promotes lats function, and a lats agonist.

106. A method for treating or preventing a disease or disorder associated with an aberrantly low level of cdc2 activity in a subject in which such treatment or prevention is desired comprising administering to the subject a therapeutically effective amount of a molecule that inhibits or antagonizes lats function.

107. The method of claim 106 in which said molecule is selected from the group consisting of a lats analog or derivative that inhibits or antagonizes lats function, an anti-lats antibody, and a *lats* antisense nucleic acid.

108. A method for screening a molecule for efficacy in treating or preventing a cancer refractory to chemotherapy or radiation therapy, said method comprising contacting cancer cells that are refractory to treatment with chemotherapeutic agents or radiation with the molecule and comparing the proliferation or survival of the contacted cells with the proliferation or survival of cells not so contacted, wherein a lower level of proliferation or survival of the contacted cells indicates that the molecule is effective to treat or prevent the cancer.

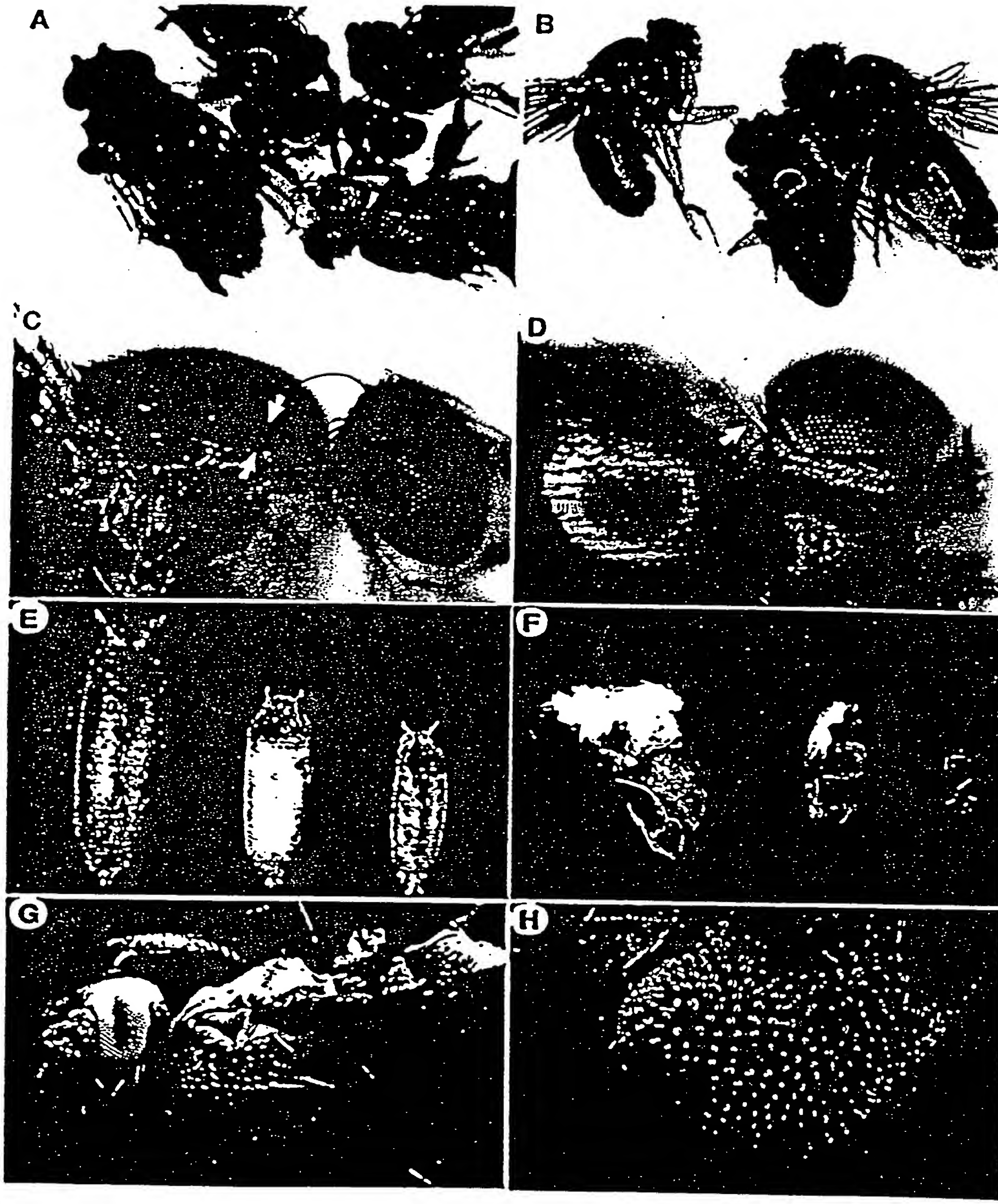
109. The method of claim 108 in which said cells are cultured *in vitro* from a tissue sample of a patient.

110. A method for screening a molecule for activity to modulate cdc2 levels or activity comprising contacting cells with the molecule, and comparing the level of cdc2 protein, mRNA or activity in cells contacted with the molecule to the amount of cdc2 protein, mRNA, or activity in cells not so contacted, wherein an increase or decrease in the amount of cdc2 protein, mRNA, or activity in the contacted cells relative to the amount of cdc2 protein, mRNA, or activity in the cells not so contacted indicates that the molecule has activity to modulate cdc2 levels or activity.

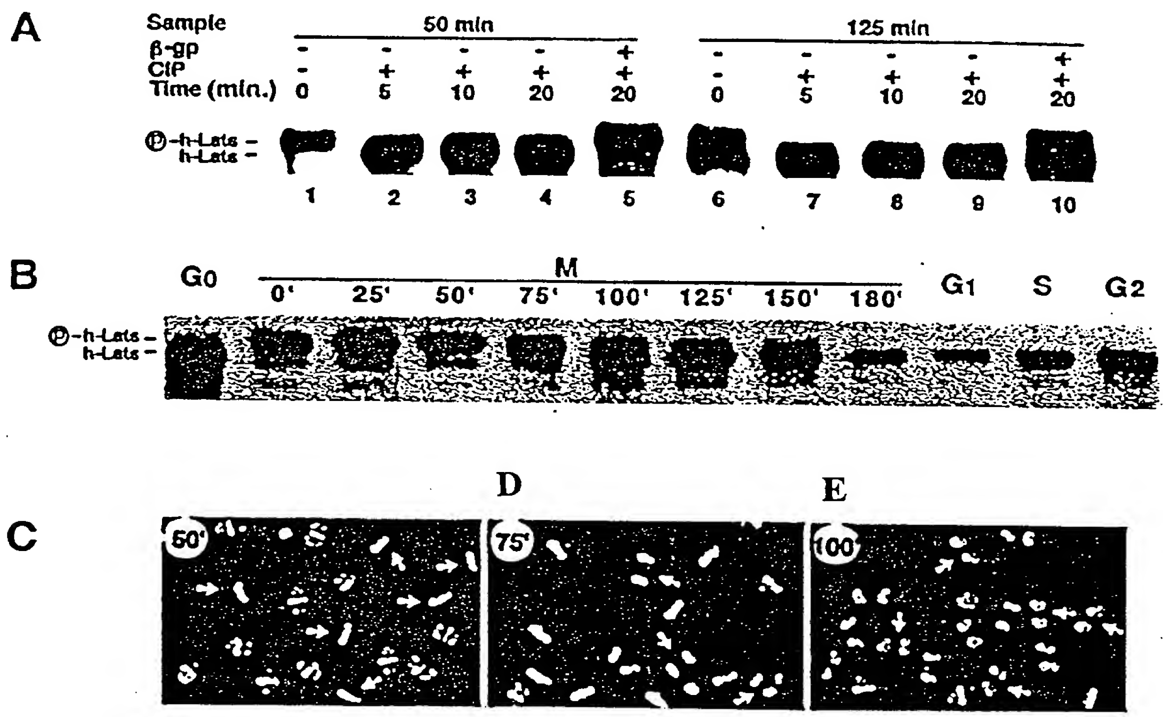
111. A method for screening a molecule for activity to modulate, directly or indirectly, the formation of a complex of lats and cdc2 proteins comprising measuring the levels of said complex formed from lats and cdc2 proteins in the presence of said molecule under conditions conducive to formation of the complex; and comparing the levels of said complex with the levels of said complex that are formed in the absence of said molecule, wherein a lower or higher level of said complex in the presence of said molecule indicates that the molecule modulates formation of said complex.

112. The method of claim 111 in which the molecule inhibits formation of the complex.

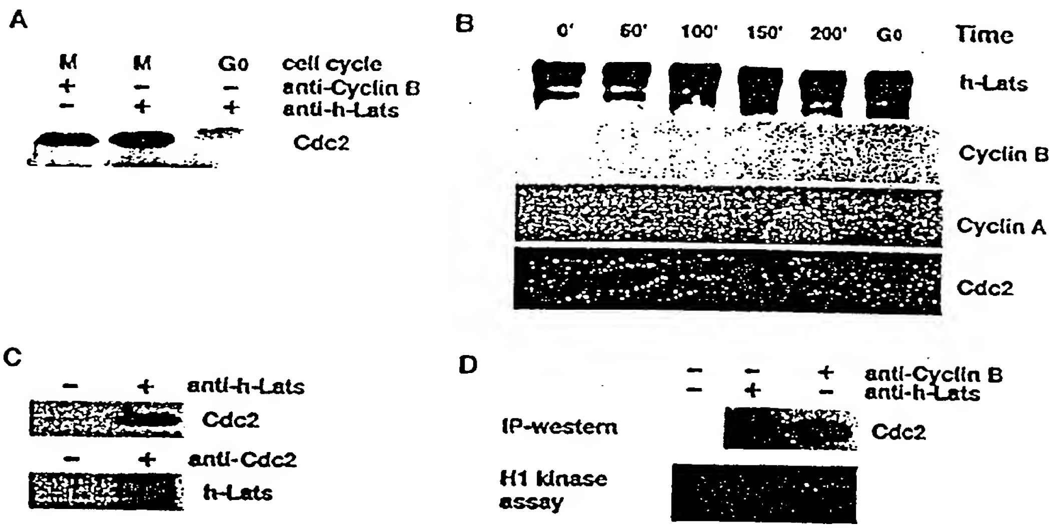
113. The method of claim 111 in which the molecule promotes formation of the complex.



FIGS. 1A-H


A
Sample
50 min
125 min
β-gp
CIP
Time (min.)
0
5
10
20
20
0
5
10
20
20
Ⓟ-h-Lats
h-Lats
1
2
3
4
5
6
7
8
9
10
B
G0
M
0'
25'
50'
75'
100'
125'
150'
180'
G1
S
G2
Ⓟ-h-Lats
h-Lats
C
D
E
50'
75'
100'

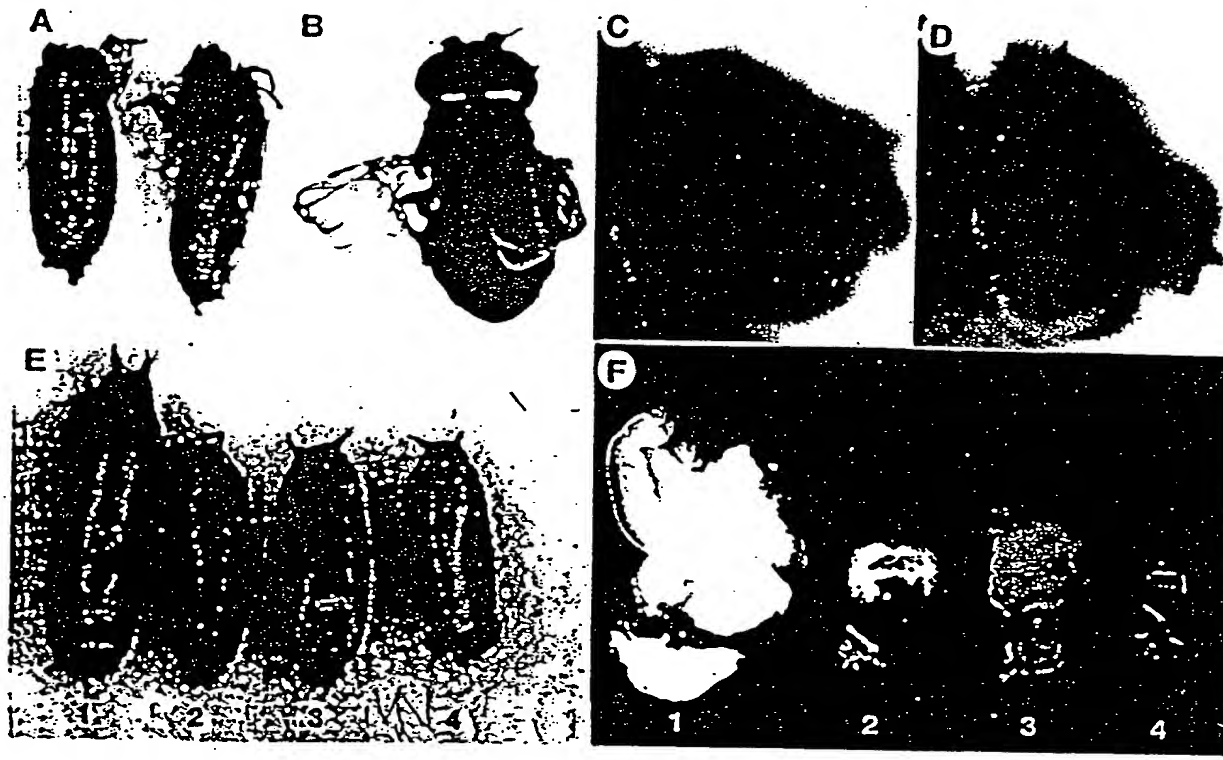
FIGS. 2A-E



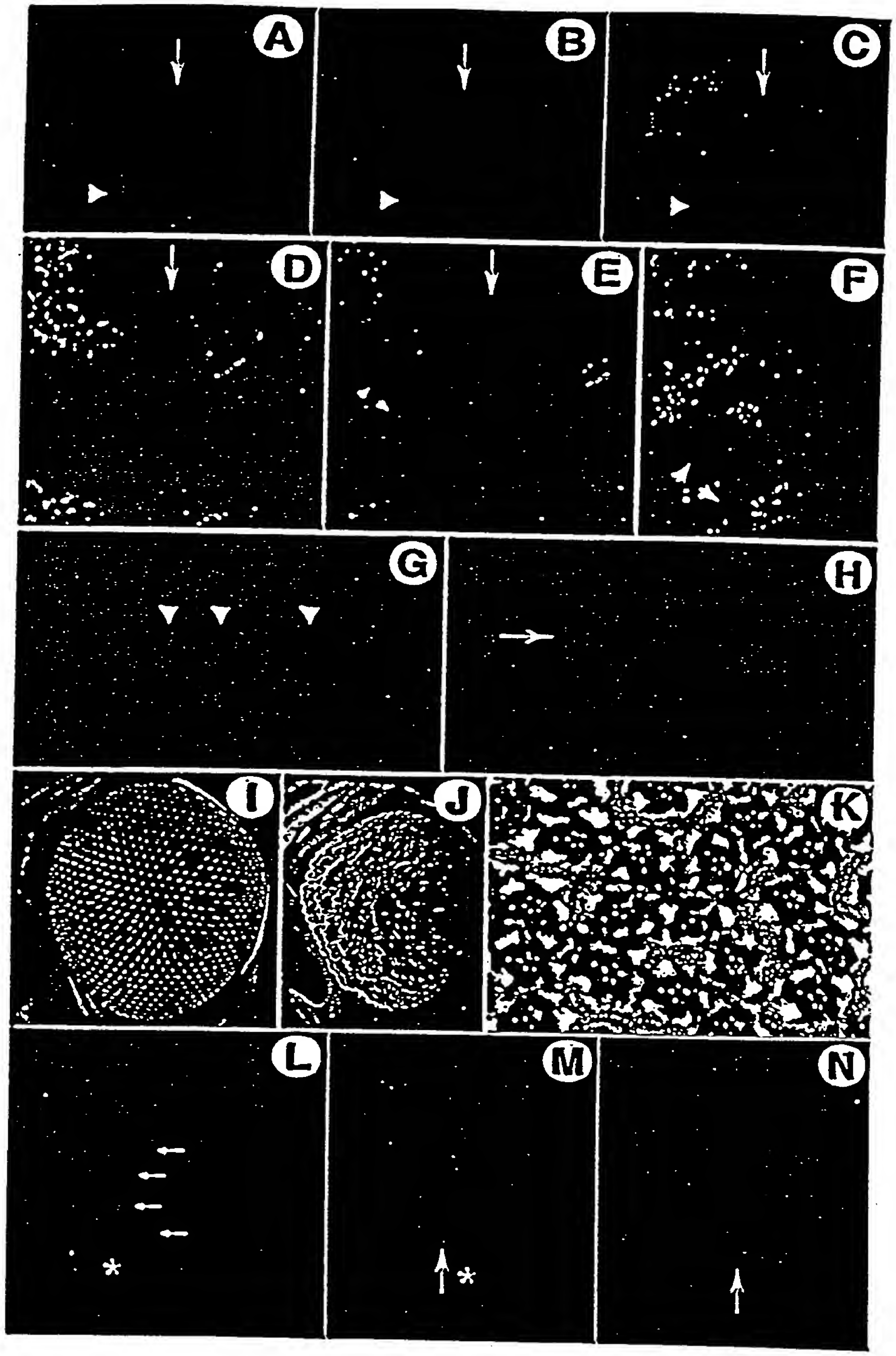
E

LexA \ gal4-AD	Cdc2	Cdk2	Cdk4	C-h-Lats
h-Lats	+	−	−	ND
N-h-Lats	+++	−	−	++
C-h-Lats	−	−	−	ND

FIGS. 3A-E



FIGS. 4A-F



FIGS. 5A-N

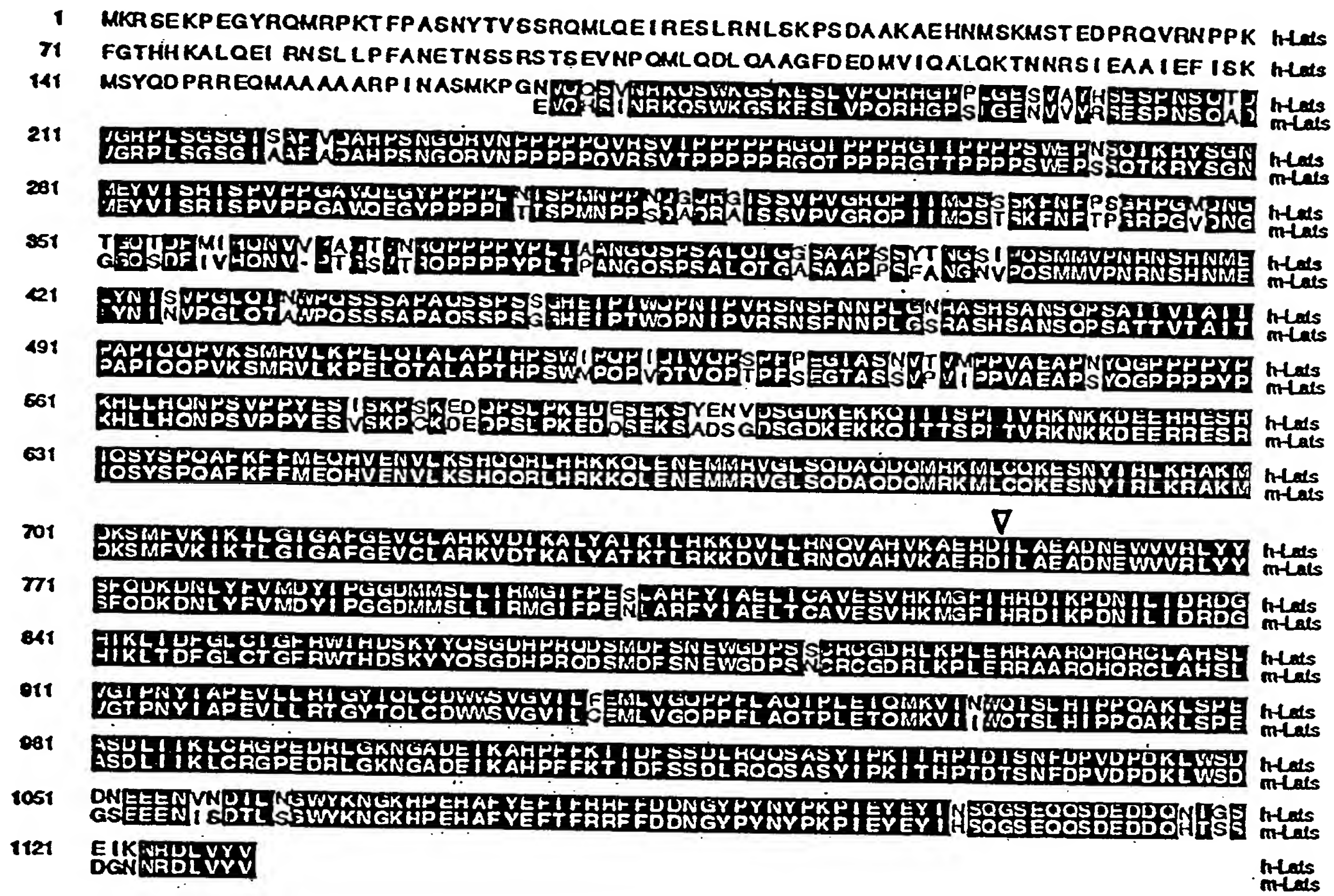
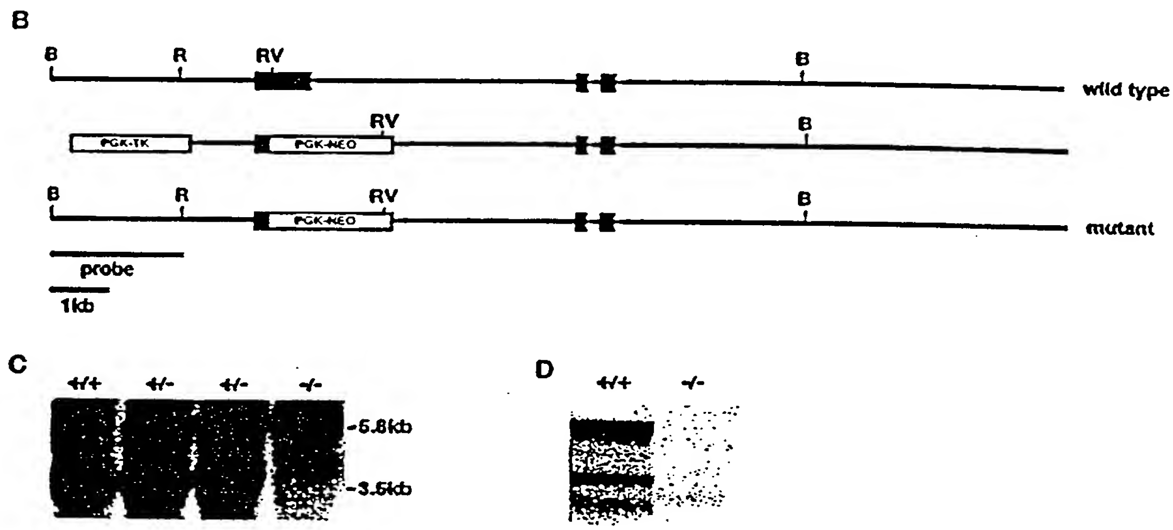
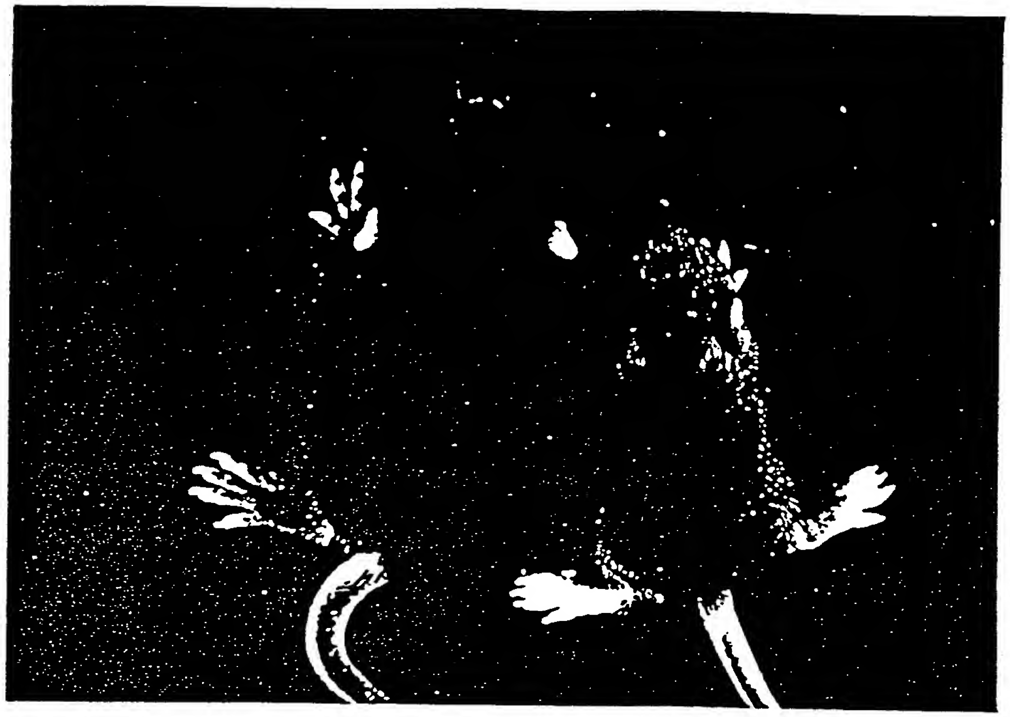

1 MKRSEKPEGYRQMRPKTFPASNYTVSSRQMLQEIRESLRNLSKPSDAAKAEHNMSKMSTEDPRQVRNPPK h-Lats
71 FGTHHKALQEIRNSLLPFANETNSSRSTSEVNPQMLQDLQAAGFDEDMVIQALQKTNNRSIEAAIEFISK h-Lats
h-Lats
m-Lats

FIG. 6A

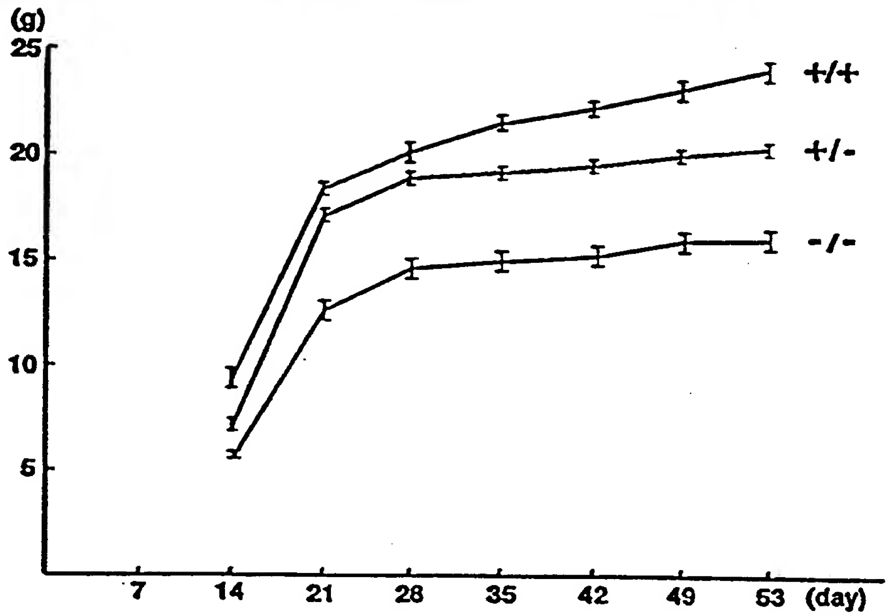

B
B
R
RV
B
wild type
RV
B
PGK-TK
PGK-NEO
B
R
RV
B
PGK-NEO
mutant
probe
1kb
C
+/+
+/-
+/-
-/-
-6.8kb
-3.5kb
D
+/+
-/-

FIGS. 6B-D

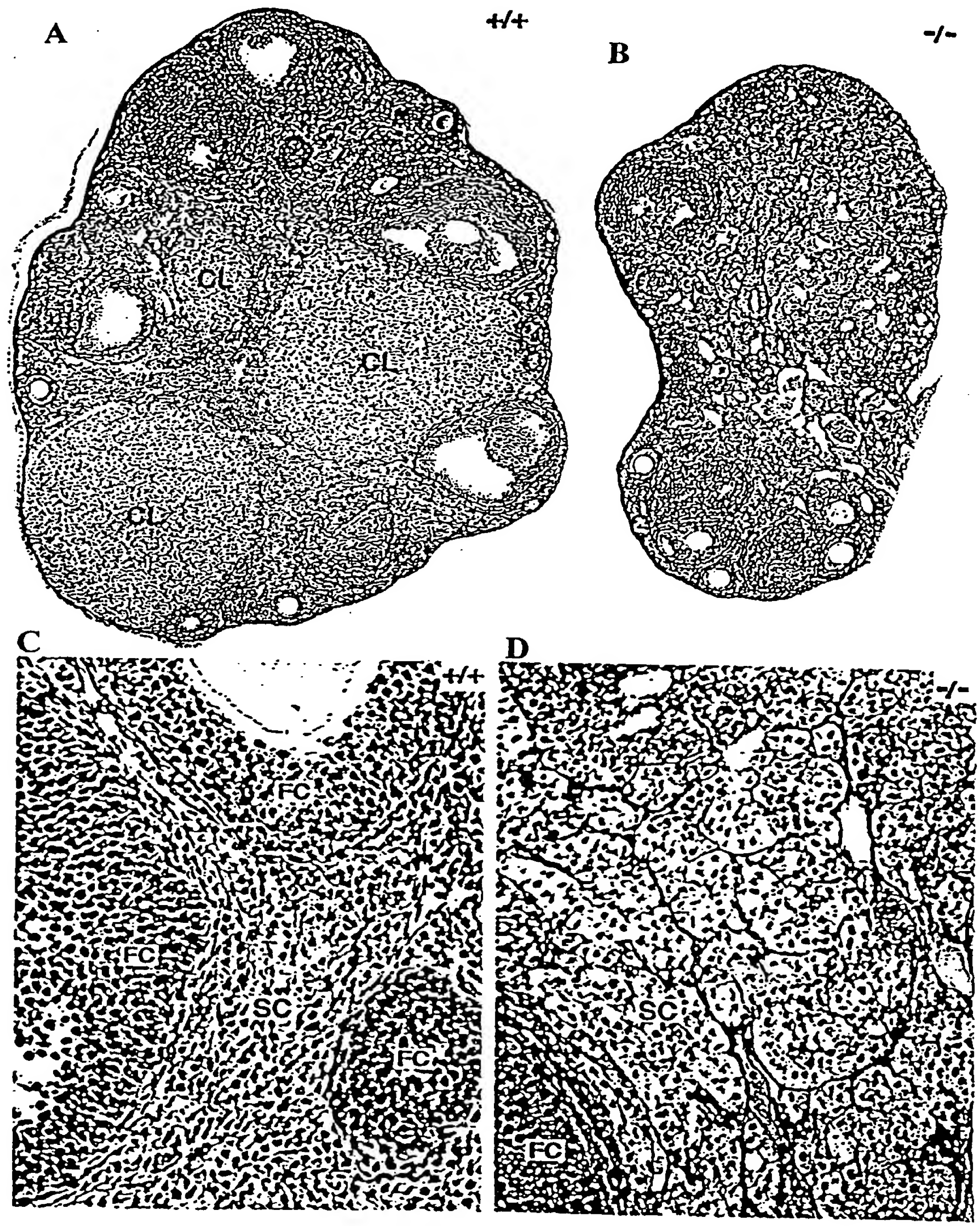
A



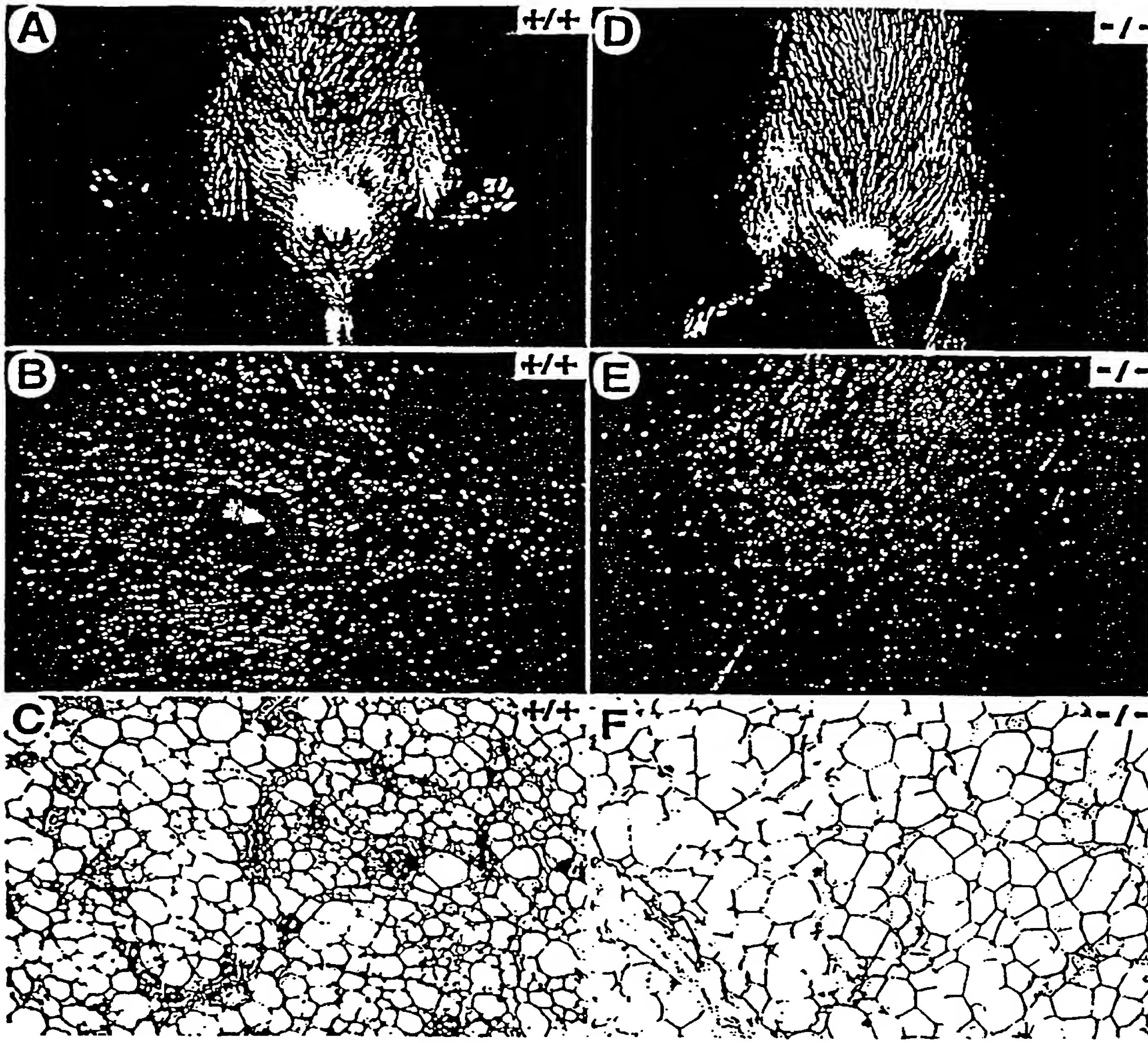
B



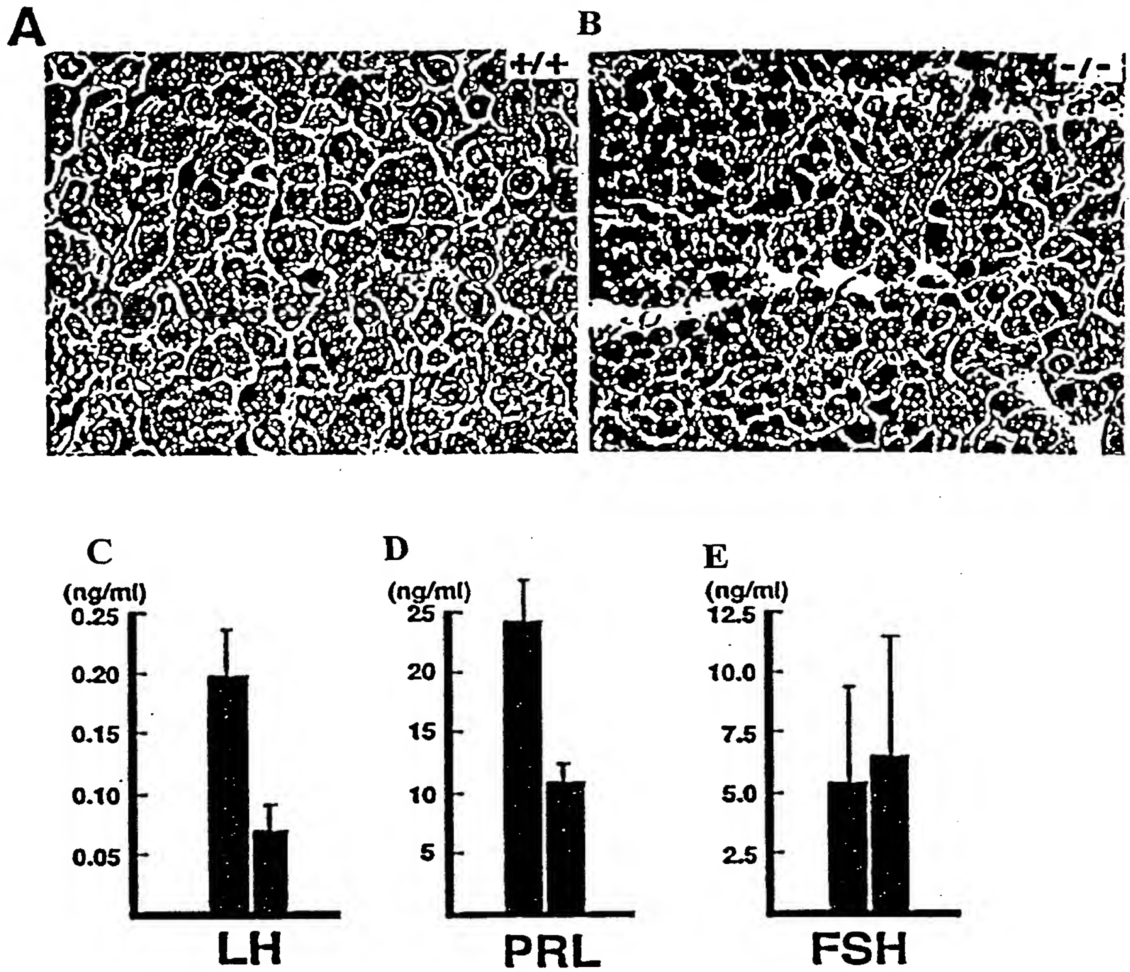
FIGS. 7A-B



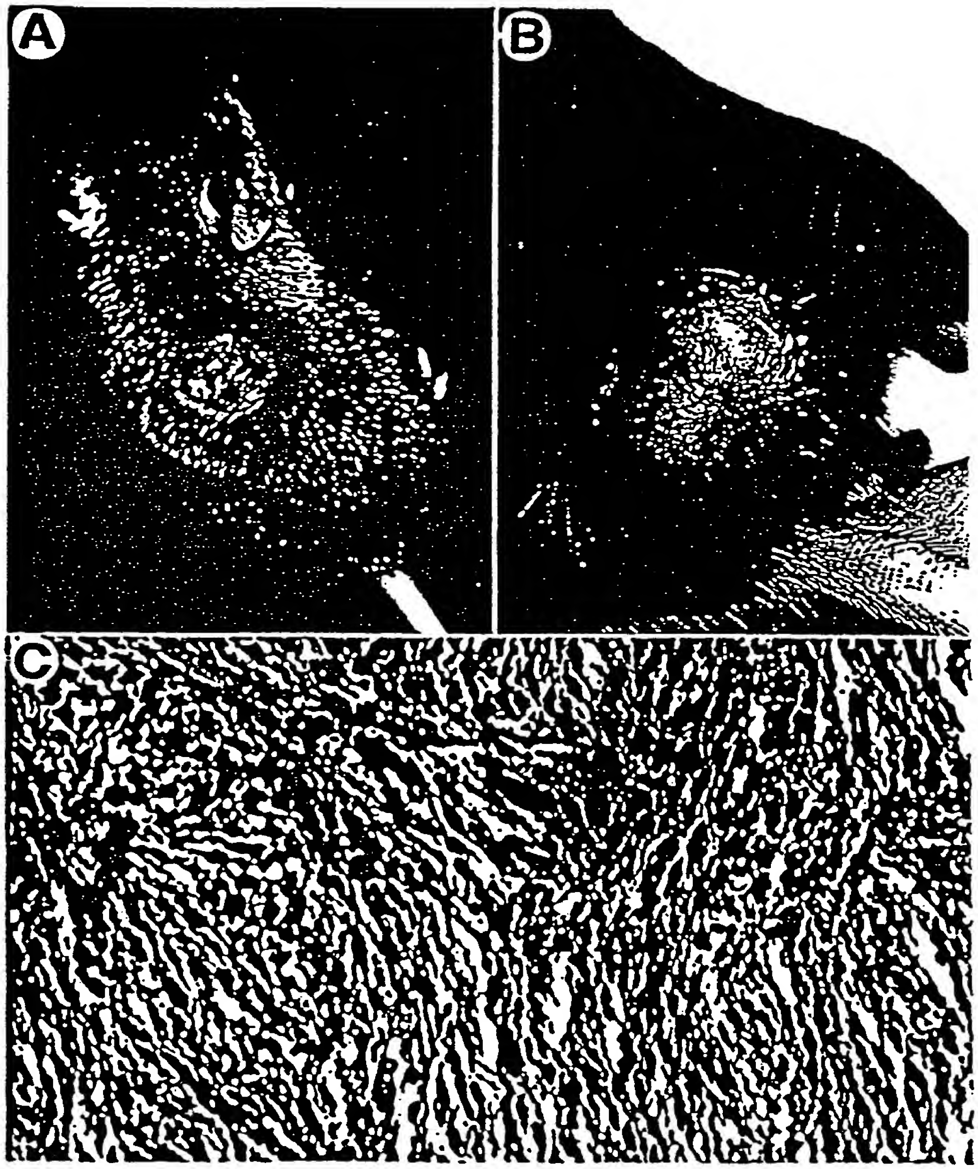
FIGS. 8A-D



FIGS. 9A-F


A
+/+
B
-/-
C
(ng/ml)
0.25
0.20
0.15
0.10
0.05
LH
D
(ng/ml)
25
20
15
10
5
PRL
E
(ng/ml)
12.5
10.0
7.5
5.0
2.5
FSH

FIGS. 10A-E



FIGS. 11A-C

```
        10         20         30         40         50         60         70         80
ACCTTTGGGT TGCTGGGACG GACTCTGGCC GCCTCAGCGT CCGCCCTCAG GCCCGTGGCC GCTGTCCAGG AGCTCTGCTC

        90        100        110        120        130        140        150        160
TCCCCTCCAG AGTTAATTAT TTATATTGTA AAGAATTTTA ACAGTCCTGG GGACTTCCTT GAAGGATCAT TTTCACTTTT

       170        180        190        200        210        220        230        240
GCTCAGAAGA AAGCTCTGGA TCTATCAAAT AAAGAAGTCC TTCGTGTGGG CTACATATAT AGATGTTTTC ATGAAGAGGA
                                                                              M  K  R

       250        260        270        280        290        300        310        320
GTGAAAAGCC AGAAGGATAT AGACAAATGA GGCCTAAGAC CTTTCCTGCC AGTAACTATA CTGTCAGTAG CCGGCAAATG
S  E  K  P   E  G  Y   R  Q  M   R  P  K  T   F  P  A   S  N  Y   T  V  S  S   R  Q  M

       330        340        350        360        370        380        390        400
TTACAAGAAA TTCGGGAATC CCTTAGGAAT TTATCTAAAC CATCTGATGC TGCTAAGGCT GAGCATAACA TGAGTAAAAT
 L  Q  E   I  R  E  S   L  R  N   L  S  K   P  S  D  A   A  K  A   E  H  N   M  S  K  M

       410        420        430        440        450        460        470        480
GTCAACCGAA GATCCTCGAC AAGTCAGAAA TCCACCCAAA TTTGGGACGC ATCATAAAGC CTTGCAGGAA ATTCGAAACT
 S  T  E   D  P  R   Q  V  R  N   P  P  K   F  G  T   H  H  K  A   L  Q  E   I  R  N

       490        500        510        520        530        540        550        560
CTCTGCTTCC ATTTGCAAAT GAAACAAATT CTTCTCGGAG TACTTCAGAA GTTAATCCAC AAATGCTTCA AGACTTGCAA
S  L  L  P   F  A  N   E  T  N   S  S  R  S   T  S  E   V  N  P   Q  M  L  Q   D  L  Q

       570        580        590        600        610        620        630        640
GCTGCTGGAT TTGATGAGGA TATGGTTATA CAAGCTCTTC AGAAAACTAA CAACAGAAGT ATAGAAGCAG CAATTGAATT
 A  A  G   F  D  E  D   M  V  I   Q  A  L   Q  K  T  N   N  R  S   I  E  A   A  I  E  F

       650        660        670        680        690        700        710        720
CATTAGTAAA ATGAGTTACC AAGATCCTCG ACGAGAGCAG ATGGCTGCAG CAGCTGCCAG ACCTATTAAT GCCAGCATGA
 I  S  K   M  S  Y   Q  D  P  R   R  E  Q   M  A  A   A  A  A  R   P  I  N   A  S  M

       730        740        750        760        770        780        790        800
AACCAGGGAA TGTGCAGCAA TCAGTTAACC GCAAACAGAG CTGGAAAGGT TCTAAAGAAT CCTTAGTTCC TCAGAGGCAT
K  P  G  N   V  Q  Q   S  V  N   R  K  Q  S   W  K  G   S  K  E   S  L  V  P   Q  R  H

       810        820        830        840        850        860        870        880
GGCCCGCCAC TAGGAGAAAG TGTGGCCTAT CATTCTGAGA GTCCCAACTC ACAGACAGAT GTAGGAAGAC CTTTGTCTGG
 G  P  P   L  G  E  S   V  A  Y   H  S  E   S  P  N  S   Q  T  D   V  G  R   P  L  S  G

       890        900        910        920        930        940        950        960
ATCTGGTATA TCAGCATTTG TTCAAGCTCA CCCTAGCAAC GGACAGAGAG TGAACCCCCC ACCACCACCT CAAGTAAGGA
 S  G  I   S  A  F   V  Q  A  H   P  S  N   G  Q  R   V  N  P  P   P  P  P   Q  V  R

       970        980        990       1000       1010       1020       1030       1040
GTGTTACTCC TCCACCACCT CCAAGAGGCC AGACTCCCCC TCCAAGAGGT ACAACTCCAC CTCCCCCTTC ATGGGAACCA
S  V  T  P   P  P  P   P  R  G   Q  T  P  P   P  R  G   T  T  P   P  P  P  S   W  E  P
```

FIG. 12

```
        1050       1060       1070       1080       1090       1100       1110       1120
           *          *          *          *          *          *          *          *
AACTCTCAAA CAAAGCGCTA TTCTGGAAAC ATGGAATACG TAATCTCCCG AATCTCTCCT GTCCCACCTG GGGCATGGCA
 N  S  Q   T  K  R  Y   S  G  N   M  E  Y   V  I  S  R   I  S  P   V  P  P  G  A  W  Q

        1130       1140       1150       1160       1170       1180       1190       1200
           *          *          *          *          *          *          *          *
AGAGGGCTAT CCTCCACCAC CTCTCAACAC TTCCCCCATG AATCCTCCTA ATCAAGGACA GAGAGGCATT AGTTCTGTTC
 E  G  Y   P  P  P   P  L  N  T   S  P  M   N  P  P   N  Q  G  Q   R  G  I   S  S  V

        1210       1220       1230       1240       1250       1260       1270       1280
           *          *          *          *          *          *          *          *
CTGTTGGCAG ACAACCAATC ATCATGCAGA GTTCTAGCAA ATTTAACTTT CCATCAGGGA GACCTGGAAT GCAGAATGGT
P  V  G  R   Q  P  I   I  M  Q   S  S  S  K   F  N  F   P  S  G   R  P  G  M   Q  N  G

        1290       1300       1310       1320       1330       1340       1350       1360
           *          *          *          *          *          *          *          *
ACTGGACAAA CTGATTTCAT GATACACCAA AATGTTGTCC CTGCTGGCAC TGTGAATCGG CAGCCACCAC CTCCATATCC
 T  G  Q   T  D  F  M   I  H  Q   N  V  V   P  A  G  T   V  N  R   Q  P  P   P  P  Y  P

        1370       1380       1390       1400       1410       1420       1430       1440
           *          *          *          *          *          *          *          *
TCTGACAGCA GCTAATGGAC AAAGCCCTTC TGCTTTACAA ACAGGGGGAT CTGCTGCTCC TTCGTCATAT ACAAATGGAA
 L  T  A   A  N  G   Q  S  P  S   A  L  Q   T  G  G   S  A  A  P   S  S  Y   T  N  G

        1450       1460       1470       1480       1490       1500       1510       1520
           *          *          *          *          *          *          *          *
GTATTCCTCA GTCTATGATG GTGCCAAACA GAAATAGTCA TAACATGGAA CTATATAACA TTAGTGTACC TGGACTGCAA
S  I  P  Q   S  M  M   V  P  N   R  N  S  H   N  M  E   L  Y  N   I  S  V  P   G  L  Q

        1530       1540       1550       1560       1570       1580       1590       1600
           *          *          *          *          *          *          *          *
ACAAATTGGC CTCAGTCATC TTCTGCTCCA GCCCAGTCAT CCCCGAGCAG TGGGCATGAA ATCCCTACAT GGCAACCTAA
 T  N  W   P  Q  S  S   S  A  P   A  Q  S   S  P  S  S   G  H  E   I  P  T   W  Q  P  N

        1610       1620       1630       1640       1650       1660       1670       1680
           *          *          *          *          *          *          *          *
CATACCAGTG AGGTCAAATT CTTTTAATAA CCCATTAGGA AATAGAGCAA GTCACTCTGC TAATTCTCAG CCTTCTGCTA
 I  P  V   R  S  N   S  F  N  N   P  L  G   N  R  A   S  H  S  A   N  S  Q   P  S  A

        1690       1700       1710       1720       1730       1740       1750       1760
           *          *          *          *          *          *          *          *
CAACAGTCAC TGCAATTACA CCAGCTCCTA TTCAACAGCC TGTGAAAAGT ATGCGTGTAT TAAAACCAGA GCTACAGACT
T  T  V  T   A  I  T   P  A  P   I  Q  Q  P   V  K  S   M  R  V   L  K  P  E   L  Q  T

        1770       1780       1790       1800       1810       1820       1830       1840
           *          *          *          *          *          *          *          *
GCTTTAGCAC CTACACACCC TTCTTGGATA CCACAGCCAA TTCAAACTGT TCAACCCAGT CCTTTTCCTG AGGGAACCGC
 A  L  A   P  T  H  P   S  W  I   P  Q  P   I  Q  T  V   Q  P  S   P  F  P   E  G  T  A

        1850       1860       1870       1880       1890       1900       1910       1920
           *          *          *          *          *          *          *          *
TTCAAATGTG ACTGTGATGC CACCTGTTGC TGAAGCTCCA AACTATCAAG GACCACCACC ACCCTACCCA AAACATCTGC
 S  N  V   T  V  M   P  P  V  A   E  A  P   N  Y  Q   G  P  P  P   P  Y  P   K  H  L

        1930       1940       1950       1960       1970       1980       1990       2000
           *          *          *          *          *          *          *          *
TGCACCAAAA CCCATCTGTT CCTCCATACG AGTCAATCAG TAAGCCTAGC AAAGAGGATC AGCCAAGCTT GCCCAAGGAA
L  H  Q  N   P  S  V   P  P  Y   E  S  I  S   K  P  S   K  E  D   Q  P  S  L   P  K  E

        2010       2020       2030       2040       2050       2060       2070       2080
           *          *          *          *          *          *          *          *
GATGAGAGTG AAAAGAGTTA TGAAAATGTT GATAGTGGGG ATAAAGAAAA GAAACAGATT ACAACTTCAC CTATTACTGT
 D  E  S   E  K  S  Y   E  N  V   D  S  G   D  K  E  K   K  Q  I   T  T  S   P  I  T  V
```

FIG. 12 (cont.)

```
2090       2100       2110       2120       2130       2140       2150       2160
TAGGAAAAAC AAGAAAGATG AAGAGCGAAG GGAATCTCGT ATTCAAAGTT ATTCTCCTCA AGCATTTAAA TTCTTTATGG
 R  K  N    K  K  D   E  E  R  R   E  S  R   I  Q  S   Y  S  P  Q   A  F  K    F  F  M

2170       2180       2190       2200       2210       2220       2230       2240
AGCAACATGT AGAAAATGTA CTCAAATCTC ATCAGCAGCG TCTACATCGT AAAAAACAAT TAGAGAATGA AATGATGCGG
E  Q  H  V   E  N  V   L  K  S   H  Q  Q  R   L  H  R   K  K  Q   L  E  N  E   M  M  R

2250       2260       2270       2280       2290       2300       2310       2320
GTTGGATTAT CTCAAGATGC CCAGGATCAA ATGAGAAAGA TGCTTTGCCA AAAAGAATCT AATTACATCC GTCTTAAAAG
 V  G  L    S  Q  D  A   Q  D  Q   M  R  K   M  L  C  Q   K  E  S   N  Y  I   R  L  K  R

2330       2340       2350       2360       2370       2380       2390       2400
GGCTAAAATG GACAAGTCTA TGTTTGTGAA GATAAAGACA CTAGGAATAG GAGCATTTGG TGAAGTCTGT CTAGCAAGAA
 A  K  M    D  K  S   M  F  V  K   I  K  T   L  G  I   G  A  F  G   E  V  C   L  A  R

2410       2420       2430       2440       2450       2460       2470       2480
AAGTAGATAC TAAGGCTTTG TATGCAACAA AAACTCTTCG AAAGAAAGAT GTTCTTCTTC GAAATCAAGT CGCTCATGTT
K  V  D  T   K  A  L   Y  A  T   K  T  L  R   K  K  D   V  L  L   R  N  Q  V   A  H  V

2490       2500       2510       2520       2530       2540       2550       2560
AAGGCTGAGA GAGATATCCT GGCTGAAGCT GACAATGAAT GGGTAGTTCG TCTATATTAT TCATTCCAAG ATAAGGACAA
 K  A  E   R  D  I  L   A  E  A   D  N  E   W  V  V  R   L  Y  Y   S  F  Q   D  K  D  N

2570       2580       2590       2600       2610       2620       2630       2640
TTTATACTTT GTAATGGACT ACATTCCTGG GGGTGATATG ATGAGCCTAT TAATTAGAAT GGGCATCTTT CCAGAAAGTC
 L  Y  F    V  M  D   Y  I  P  G   G  D  M   M  S  L   L  I  R  M   G  I  F   P  E  S

2650       2660       2670       2680       2690       2700       2710       2720
TGGCACGATT CTACATAGCA GAACTTACCT GTGCAGTTGA AAGTGTTCAT AAAATGGGTT TTATTCATAG AGATATTAAA
L  A  R  F   Y  I  A   E  L  T   C  A  V  E   S  V  H   K  M  G   F  I  H  R   D  I  K

2730       2740       2750       2760       2770       2780       2790       2800
CCTGATAATA TTTTGATTGA TCGTGATGGT CATATTAAAT TGACTGACTT TGGCCTCTGC ACTGGCTTCA GATGGACACA
 P  D  N   I  L  I  D   R  D  G   H  I  K   L  T  D  F   G  L  C   T  G  F   R  W  T  H

2810       2820       2830       2840       2850       2860       2870       2880
CGATTCTAAG TACTATCAGA GTGGTGACCA TCCACGGCAA GATAGCATGG ATTTCAGTAA TGAATGGGGG GATCCCTCAA
 D  S  K    Y  Y  Q   S  G  D  H   P  R  Q   D  S  M   D  F  S  N   E  W  G   D  P  S

2890       2900       2910       2920       2930       2940       2950       2960
GCTGTCGATG TGGAGACAGA CTGAAGCCAT TAGAGCGGAG AGCTGCACGC CAGCACCAGC GATGTCTAGC ACATTCTTTG
S  C  R  C   G  D  R   L  K  P   L  E  R  R   A  A  R   Q  H  Q   R  C  L  A   H  S  L

2970       2980       2990       3000       3010       3020       3030       3040
GTTGGGACTC CCAATTATAT TGCACCTGAA GTGTTGCTAC GAACAGGATA CACACAGTTG TGTGATTGGT GGAGTGTTGG
 V  G  T   P  N  Y  I   A  P  E   V  L  L   R  T  G  Y   T  Q  L   C  D  W   W  S  V  G

3050       3060       3070       3080       3090       3100       3110       3120
TGTTATTCTT TTTGAAATGT TGGTGGGACA ACCTCCTTTC TTGGCACAAA CACCATTAGA AACACAAATG AAGGTTATCA
 V  I  L    F  E  M   L  V  G  Q   P  P  F   L  A  Q   T  P  L  E   T  Q  M   K  V  I
```

FIG. 12 (cont.)

```
      3130       3140       3150       3160       3170       3180       3190       3200
ACTGGCAAAC ATCTCTTCAC ATTCCACCAC AAGCTAAACT CAGTCCTGAA GCTTCTGATC TTATTATTAA ACTTTGCCGA
N  W  Q  T   S  L  H   I  P  P   Q  A  K  L   S  P  E   A  S  D   L  I  I  K   L  C  R

      3210       3220       3230       3240       3250       3260       3270       3280
GGACCCGAAG ATCGCTTAGG CAAGAATGGT GCTGATGAAA TAAAAGCTCA TCCATTTTTT AAAACAATTG ACTTCTCCAG
 G  P  E   D  R  L  G   K  N  G   A  D  E   I  K  A  H   P  F  F   K  T  I   D  F  S  S

      3290       3300       3310       3320       3330       3340       3350       3360
TGACCTGAGA CAGCAGTCTG CTTCATACAT TCCTAAAATC ACACACCCAA CAGATACATC AAATTTTGAT CCTGTTGATC
 D  L  R   Q  Q  S   A  S  Y  I   P  K  I   T  H  P   T  D  T  S   N  F  D   P  V  D

      3370       3380       3390       3400       3410       3420       3430       3440
CTGATAAATT ATGGAGTGAT GATAACGAGG AAGAAAATGT AAATGACACT CTCAATGGAT GGTATAAAAA TGGAAAGCAT
P  D  K  L   W  S  D   D  N  E   E  E  N  V   N  D  T   L  N  G   W  Y  K  N   G  K  H

      3450       3460       3470       3480       3490       3500       3510       3520
CCTGAACATG CATTCTATGA ATTTACCTTC CGAAGGTTTT TTGATGACAA TGGCTACCCA TATAATTATC CGAAGCCTAT
 P  E  H   A  F  Y  E   F  T  F   R  R  F   F  D  D  N   G  Y  P   Y  N  Y   P  K  P  I

      3530       3540       3550       3560       3570       3580       3590       3600
TGAATATGAA TACATTAATT CACAAGGCTC AGAGCAGCAG TCGGATGAAG ATGATCAAAA CACAGGCTCA GAGATTAAAA
 E  Y  E   Y  I  N   S  Q  G  S   E  Q  Q   S  D  E   D  D  Q  N   T  G  S   E  I  K

      3610       3620       3630       3640       3650       3660       3670       3680
ATCGCGATCT AGTATATGTT TAACACACTA GTAAATAAAT GTAATGAGGA TTTGTAAAAG GGCCTGAAAT GCGAGGTGTT
N  R  D  L   V  Y  V   *

      3690       3700       3710       3720       3730       3740       3750       3760
TTGAGGTTCT GAGAGTAAAA TTATGCAAAT ATGACAGAGC TATATATGTG TGCTCTGTGT ACAATATTTT ATTTTCCTAA

      3770       3780       3790       3800       3810       3820       3830       3840
ATTATGGGAA ATCCTTTTAA AATGTTAATT TATTCCAGCC GTTTAAATCA GTATTTAGAA AAAAATTGTT ATAAGGAAAG

      3850       3860       3870       3880       3890       3900       3910       3920
TAAATTATGA ACTGAATATT ATAGTCAGTT CTTGGTACTT AAAGTACTTA AAATAAGTAG TGCTTTGTTT AAAAGGAGAA

      3930       3940       3950       3960       3970       3980
ACCTGGTATC TATTTGTATA TATGCTAAAT AATTTTAAAA TACAAGAGTT TTTGAAATTT TTTT
```

FIG. 12 (cont.)

```
        10         20         30         40         50         60         70         80
GTGCAACATT CAATTAACCG AAAACAAAGC TGGAAAGGTT CTAAAGAGTC TCTAGTTCCT CAGAGACACG GCCCATCTCT
 V  Q  H   S  I  N  R  K  Q  S  W  K  G   S  K  E  S  L  V  P   Q  R  H  G  P  S  L

        90        100        110        120        130        140        150        160
AGGAGAAAAT GTGGTTTATC GTTCTGAAAG CCCCAACTCA CAGGCGGATG TAGGAAGACC TCTGTCTGGA TCCGGCATTG
  G  E  N   V  V  Y   R  S  E  S   P  N  S   Q  A  D  V  G  R  P   L  S  G   S  G  I

       170        180        190        200        210        220        230        240
CAGCATTTGC TCAAGCTCAC CCAAGCAATG GACAGAGAGT GAACCCCCCA CCACCACCTC AAGTTAGGAG TGTTACTCCT
A  A  F  A  Q  A  H   P  S  N  G  Q  R  V   N  P  P   P  P  P  Q  V  R  S   V  T  P

       250        260        270        280        290        300        310        320
CCACCACCTC CGAGAGGCCA GACCCCACCT CCCCGAGGCA CCACTCCCCC TCCCCCCTCA TGGGAACCAA GCTCTCAGAC
 P  P  P   P  R  G  Q   T  P  P   P  R  G   T  T  P  P   P  P  S   W  E  P   S  S  Q  T

       330        340        350        360        370        380        390        400
AAAGCGCTAC TCTGGGAACA TGGAGTACGT AATCTCCCGA ATCTCCCCTG TTCCACCTGG GGCGTGGCAG GAGGGGTACC
 K  R  Y   S  G  N   M  E  Y  V   I  S  R   I  S  P   V  P  P  G   A  W  Q   E  G  Y

       410        420        430        440        450        460        470        480
CTCCACCACC TCTTACCACT TCTCCCATGA ATCCCCCTAG CCAGGCTCAG AGGGCCATTA GTTCTGTTCC AGTTGGTAGA
P  P  P  P  L  T  T   S  P  M   N  P  P  S   Q  A  Q   R  A  I   S  S  V  P   V  G  R

       490        500        510        520        530        540        550        560
CAACCCATCA TCATGCAGAG TACTAGCAAA TTTAACTTTA CACCAGGGCG ACCTGGAGTT CAGAATGGTG GTGGTCAGTC
 Q  P  I   I  M  Q  S   T  S  K   F  N  F   T  P  G  R   P  G  V   Q  N  G   G  G  Q  S

       570        580        590        600        610        620        630        640
TGATTTTATC GTGCACCAAA ATGTCCCCAC TGGTTCTGTG ACTCGGCAGC CACCACCTCC ATATCCTCTG ACCCCAGCTA
  D  F  I   V  H  Q   N  V  P  T   G  S  V   T  R  Q   P  P  P  P   Y  P  L   T  P  A

       650        660        670        680        690        700        710        720
ATGGACAAAG CCCCTCTGCT TTACAAACAG GGGCTTCTGC TGCTCCACCA TCATTCGCCA ATGGAAACGT TCCTCAGTCG
N  G  Q  S   P  S  A   L  Q  T   G  A  S  A   A  P  P   S  F  A   N  G  N  V   P  Q  S

       730        740        750        760        770        780        790        800
ATGATGGTGC CCAACAGGAA CAGTCATAAC ATGGAGCTTT ATAATATTAA TGTCCCTGGA CTGCAAACAG CCTGGCCCCA
 M  M  V   P  N  R  N   S  H  N   M  E  L   Y  N  I  N   V  P  G   L  Q  T   A  W  P  Q

       810        820        830        840        850        860        870        880
GTCGTCTTCT GCTCCTGCGC AGTCATCCCC AAGCGGTGGG CATGAAATTC CTACATGGCA ACCTAACATA CCAGTGAGGT
 S  S  S   A  P  A   Q  S  S  P   S  G  G   H  E  I   P  T  W  Q   P  N  I   P  V  R

       890        900        910        920        930        940        950        960
CAAATTCTTT TAATAACCCA TTAGGAAGTA GAGCAAGTCA CTCTGCTAAT TCTCAGCCTT CTGCCACTAC AGTCACTGCC
S  N  S  F   N  N  P   L  G  S   R  A  S  H   S  A  N   S  Q  P   S  A  T  T   V  T  A
```

FIG. 13

970 980 990 1000 1010 1020 1030 1040
ATCACACCCG CTCCTATTCA ACAGCCCGTG AAAAGCATGC GCGTCCTGAA ACCAGAGCTG CAGACTGCTT TAGCCCCAAC
I T P A P I Q Q P V K S M R V L K P E L Q T A L A P T

1050 1060 1070 1080 1090 1100 1110 1120
CCATCCTTCT TGGATGCCAC AGCCAGTTCA GACTGTTCAG CCTACCCCTT TTTCTGAGGG TACAGCTTCA AGTGTGCCTG
H P S W M P Q P V Q T V Q P T P F S E G T A S S V P

1130 1140 1150 1160 1170 1180 1190 1200
TCATCCCACC TGTTGCTGAA GCTCCAAGCT ATCAAGGTCC ACCACCGCCT TATCCAAAAC ATCTGCTACA CCAAAACCCA
V I P P V A E A P S Y Q G P P P P Y P K H L L H Q N P

1210 1220 1230 1240 1250 1260 1270 1280
TCTGTCCCTC CATATGAGTC AGTAAGTAAG CCCTGCAAAG ATGAACAGCC TAGCTTACCC AAGGAAGATG ATAGTGAGAA
S V P P Y E S V S K P C K D E Q P S L P K E D D S E K

1290 1300 1310 1320 1330 1340 1350 1360
GAGTGCGGAC AGTGGTGACT CTGGGGATAA AGAAAAGAAA CAGATTACAA CTTCACCTAT CACTGTTCGG AAAAACAAGA
S A D S G D S G D K E K K Q I T T S P I T V R K N K

1370 1380 1390 1400 1410 1420 1430 1440
AAGATGAAGA ACGAAGAGAG TCTCGGATTC AGAGTTACTC CCCACAGGCC TTTAAGTTCT TCATGGAGCA GCACGTAGAG
K D E E R R E S R I Q S Y S P Q A F K F F M E Q H V E

1450 1460 1470 1480 1490 1500 1510 1520
AACGTCCTGA AGTCTCATCA GCAGCGTCTG CATCGGAAGA AGCAGCTAGA AAATGAAATG ATGCGGGTTG GATTATCTCA
N V L K S H Q Q R L H R K K Q L E N E M M R V G L S Q

1530 1540 1550 1560 1570 1580 1590 1600
AGATGCCCAG GATCAAATGA GAAAGATGCT TTGCCAGAAA GAGTCTAACT ATATTCGTCT TAAAAGGGCT AAAATGGACA
D A Q D Q M R K M L C Q K E S N Y I R L K R A K M D

1610 1620 1630 1640 1650 1660 1670 1680
AGTCTATGTT TGTAAAGATA AAGACATTAG GAATAGGAGC GTTTGGTGAA GTCTGTCTAG CAAGAAAAGT CGATACTAAA
K S M F V K I K T L G I G A F G E V C L A R K V D T K

1690 1700 1710 1720 1730 1740 1750 1760
GCTTTGTATG CAACAAAGAC TCTTCGAAAG AAAGACGTTC TGCTCCGAAA TCAGGTGGCT CATGTGAAAG CGGAGAGGGA
A L Y A T K T L R K K D V L L R N Q V A H V K A E R D

1770 1780 1790 1800 1810 1820 1830 1840
TATCCTAGCA GAAGCCGACA ATGAGTGGGT GGTCCGCCTG TACTACTCTT TCCAGGACAA GGACAACTTG TACTTTGTGA
I L A E A D N E W V V R L Y Y S F Q D K D N L Y F V

1850 1860 1870 1880 1890 1900 1910 1920
TGGACTACAT TCCTGGGGGG GATATGATGA GCCTATTAAT TAGAATGGGC ATCTTTCCTG AAAATCTGGC ACGATTCTAC
M D Y I P G G D M M S L L I R M G I F P E N L A R F Y

1930 1940 1950 1960 1970 1980 1990 2000

FIG. 13 (cont.)

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ATAGCAGAAC TTACCTGTGC AGTTGAAAGT GTTCATAAAA TGGGTTTTAT TCATAGAGAT ATTAAACCTG ATAACATTTT
 I  A  E  L  T  C  A  V  E  S  V  H  K  M  G  F  I  H  R  D  I  K  P  D  N  I  L

      2010       2020       2030       2040       2050       2060       2070       2080
         *          *          *          *          *          *          *          *
GATTGACCGT GATGGCCATA TTAAATTGAC TGACTTTGGC TTGTGCACTG GCTTCAGATG GACACATGAC TCCAAGTACT
 I  D  R  D  G  H  I  K  L  T  D  F  G  L  C  T  G  F  R  W  T  H  D  S  K  Y

      2090       2100       2110       2120       2130       2140       2150       2160
         *          *          *          *          *          *          *          *
ACCAGAGTGG GGATCACCCA CGGCAAGATA GCATGGATTT CAGTAACGAA TGGGGAGATC CTTCCAATTG TCGGTGTGGG
Y  Q  S  G  D  H  P  R  Q  D  S  M  D  F  S  N  E  W  G  D  P  S  N  C  R  C  G

      2170       2180       2190       2200       2210       2220       2230       2240
         *          *          *          *          *          *          *          *
GACAGACTGA AGCCACTGGA GCGGAGAGCT GCTCGCCAGC ACCAGCGATG TCTAGCCCAT TCTCTGGTTG GGACTCCCAA
 D  R  L  K  P  L  E  R  R  A  A  R  Q  H  Q  R  C  L  A  H  S  L  V  G  T  P  N

      2250       2260       2270       2280       2290       2300       2310       2320
         *          *          *          *          *          *          *          *
TTATATTGCA CCTGAAGTGC TACTGCGAAC AGGATATACA CAGCTGTGTG ACTGGTGGAG TGTTGGTGTT ATTCTTTGTG
  Y  I  A  P  E  V  L  L  R  T  G  Y  T  Q  L  C  D  W  W  S  V  G  V  I  L  C

      2330       2340       2350       2360       2370       2380       2390       2400
         *          *          *          *          *          *          *          *
AAATGTTGGT GGGACAACCT CCTTTCTTGG CACAAACCCC ATTAGAAACA CAAATGAAGG TTATCATCTG GCAAACTTCT
E  M  L  V  G  Q  P  P  F  L  A  Q  T  P  L  E  T  Q  M  K  V  I  I  W  Q  T  S

      2410       2420       2430       2440       2450       2460       2470       2480
         *          *          *          *          *          *          *          *
CTACACATCC CTCCTCAAGC TAAGCTGAGT CCTGAAGCCT CTGACCTCAT TATCAAACTG TGTCGAGGAC CAGAAGACCG
 L  H  I  P  P  Q  A  K  L  S  P  E  A  S  D  L  I  I  K  L  C  R  G  P  E  D  R

      2490       2500       2510       2520       2530       2540       2550       2560
         *          *          *          *          *          *          *          *
CCTCGGCAAG AACGGTGCTG ATGAGATAAA GGCTCATCCA TTTTTTAAGA CCATCGATTT CTCTAGTGAT CTGAGACAGC
  L  G  K  N  G  A  D  E  I  K  A  H  P  F  F  K  T  I  D  F  S  S  D  L  R  Q

      2570       2580       2590       2600       2610       2620       2630       2640
         *          *          *          *          *          *          *          *
AGTCTGCTTC ATACATCCCT AAAATCACGC ATCCAACAGA TACATCCAAT TTCGACCCTG TTGATCCTGA TAAATTGTGG
Q  S  A  S  Y  I  P  K  I  T  H  P  T  D  T  S  N  F  D  P  V  D  P  D  K  L  W

      2650       2660       2670       2680       2690       2700       2710       2720
         *          *          *          *          *          *          *          *
AGCGATGGCA GCGAGGAGGA AAATATCAGT GACACTCTGA GCGGATGGTA TAAAAATGGG AAGCACCCCG AGCACGCTTT
 S  D  G  S  E  E  E  N  I  S  D  T  L  S  G  W  Y  K  N  G  K  H  P  E  H  A  F

      2730       2740       2750       2760       2770       2780       2790       2800
         *          *          *          *          *          *          *          *
CTATGAGTTC ACCTTTCGGA GGTTTTTTGA TGACAATGGC TACCCATATA ATTATCCAAA GCCTATTGAG TATGAATACA
  Y  E  F  T  F  R  R  F  F  D  D  N  G  Y  P  Y  N  Y  P  K  P  I  E  Y  E  Y

      2810       2820       2830       2840       2850       2860       2870       2880
         *          *          *          *          *          *          *          *
TTCATTCACA GGGCTCAGAA CAACAGTCTG ATGAAGATGA TCAACACACA AGCTCCGATG GAAACAACCG AGATCTAGTG
I  H  S  Q  G  S  E  Q  Q  S  D  E  D  D  Q  H  T  S  S  D  G  N  N  R  D  L  V

      2890       2900       2910       2920       2930       2940       2950       2960
         *          *          *          *          *          *          *          *
TATGTTTAAT AAACTAGGAG ATCATTGTAA GAATTTGCAA GAGGCCTGAA GTGCAGGGGT TTTTGAAGTT TTGAGAAAAT
  Y  V  *
```

FIG. 13 (cont.)

```
      2970       2980       2990       3000       3010       3020       3030       3040
         *          *          *          *          *          *          *          *
TATGCAAATG TGACAGAGTT TGTGTGCTCT GTGTACAATA TTTTATTTTC CTAAGTTATG GGAAATTGTT TTAAAATGTT

      3050       3060       3070       3080       3090       3100       3110       3120
         *          *          *          *          *          *          *          *
AATTTATTCC ACCCTTTTAA TTCAGTAATT TAGAAAAAAT TGTTATAAGG AAAGTAAATT ATGAACTGAG TATTATAGTC

      3130       3140       3150       3160       3170       3180       3190       3200
         *          *          *          *          *          *          *          *
AATTCTTGGT ACTTAAAGTA CTTAAAAAGA GAAGCCTGGT ATCTTTTGTA TATATAATAA ATAATTTTAA AATCCCAAAA

      3210
         *
AAAAAAAAAA AAA
```

FIG. 13 (cont.)

```
        10         20         30         40         50         60         70         80
         *          *          *          *          *          *          *          *
ATGAGAGCCA CCCCGAAGTT TGGACCTTAT CAAAAAGCTC TCAGGGAAAT CCGATATTCC CTCCTGCCTT TTGCCAACGA
 M  R  A   T  P  K  F   G  P  Y   Q  K  A   L  R  E  I   R  Y  S   L  L  P   F  A  N  E

        90        100        110        120        130        140        150        160
         *          *          *          *          *          *          *          *
GTCAGGCACT TCGGCAGCTG CAGAGGTGAA CCGGCAGATG CTTCAGGAGT TGGTGAATGC GGCATGTGAC CAGGAGATGG
  S  G  T   S  A  A   A  E  V  N   R  Q  M   L  Q  E   L  V  N  A   A  C  D   Q  E  M

       170        180        190        200        210        220        230        240
         *          *          *          *          *          *          *          *
CTGGCAGAGC GCTCACGCAG ACGGGCAGTA GGAGTATCGA AGCTGCCTTG GAGTACATCA GTAAGATGGG CTACCTGGAC
A  G  R  A   L  T  Q   T  G  S   R  S  I  E   A  A  L   E  Y  I   S  K  M  G   Y  L  D

       250        260        270        280        290        300        310        320
         *          *          *          *          *          *          *          *
CCCAGGAATG AGCAGATTGT GCGAGTCATC AAGCAGACCT CCCCAGGAAA GGGCCTGGCG TCCACCCCGG TGACTCGGCG
 P  R  N   E  Q  I  V   R  V  I   K  Q  T   S  P  G  K   G  L  A   S  T  P   V  T  R  R

       330        340        350        360        370        380        390        400
         *          *          *          *          *          *          *          *
GCCCAGTTTC GAGGGCACAG GGGAAGCACT CCCATCCTAC CACCAGCTGG GTGGTGCAAA CTACGAGGGC CCCGCCGCAC
  P  S  F   E  G  T   G  E  A  L   P  S  Y   H  Q  L   G  G  A  N   Y  E  G   P  A  A

       410        420        430        440        450        460        470        480
         *          *          *          *          *          *          *          *
TGGAGGAGAT GCCGCGGCAA TATTTAGACT TTCTCTTCCC TGGAGCCGGA GCCGGCACCC ACGGTGCCCA GGCTCACCAG
L  E  E  M   P  R  Q   Y  L  D   F  L  F  P   G  A  G   A  G  T   H  G  A  Q   A  H  Q

       490        500        510        520        530        540        550        560
         *          *          *          *          *          *          *          *
CATCCTCCCA AAGGGTACAG CACAGCAGTA GAGCCAAGTG CGCACTTTCC GGGCACACAC TATGGTCGTG GTCATCTACT
H  P  P   K  G  Y  S   T  A  V   E  P  S   A  H  F  P   G  T  H   Y  G  R   G  H  L  L

       570        580        590        600        610        620        630        640
         *          *          *          *          *          *          *          *
ATCGGAGCAG TCTGGGTATG GGGTGCAGCG CAGTTCCTCC TTCCAGAACA AGACGCCACC AGATGCCTAT TCCAGCATGG
  S  E  Q   S  G  Y   G  V  Q  R   S  S  S   F  Q  N   K  T  P  P   D  A  Y   S  S  M

       650        660        670        680        690        700        710        720
         *          *          *          *          *          *          *          *
CCAAGGCCCA GGGTGGCCCT CCCGCCAGCC TCACCTTTCC TGCCCATGCT GGGCTGTACA CTGCCTCGCA CCACAAGCCG
A  K  A  Q   G  G  P   P  A  S   L  T  F  P   A  H  A   G  L  Y   T  A  S  H   H  K  P

       730        740        750        760        770        780        790        800
         *          *          *          *          *          *          *          *
GCGGCTACCC CACCTGGGGC CCACCCATTA CATGTGTTGG GCACCCGGGG TCCCACGTTT ACTGGCGAAA GCTCTGCACA
 A  A  T   P  P  G  A   H  P  L   H  V  L   G  T  R  G   P  T  F   T  G  E   S  S  A  Q

       810        820        830        840        850        860        870        880
         *          *          *          *          *          *          *          *
GGCTGTGCTG GCACCGTCCA GGAACAGCCT CAATGCTGAC TTGTACGAGC TGGGCTCCAC GGTGCCCTGG TCTGCAGCTC
  A  V  L   A  P  S   R  N  S  L   N  A  D   L  Y  E   L  G  S  T   V  P  W   S  A  A

       890        900        910        920        930        940        950        960
         *          *          *          *          *          *          *          *
CACTGGCACG CCGCGACTCG CTGCAGAAGC AGGGTCTAGA AGCCTCGCGG CCGCATGTGG CTTTTCGGGC TGGCCCCAGC
P  L  A  R   R  D  S   L  Q  K   Q  G  L  E   A  S  R   P  H  V   A  F  R  A   G  P  S
```

FIG. 14

```
       970        980        990       1000       1010       1020       1030       1040
AGGACCAACT CCTTCAACAA CCCACAACCT GAGCCCTCAC TGCCCGCCCC CAACACGGTC ACCGCCGTGA CGGCCGCACA
 R  T  N   S  F  N  N   P  Q  P   E  P  S   L  P  A  P   N  T  V   T  A  V   T  A  A  H

      1050       1060       1070       1080       1090       1100       1110       1120
CATCCTTCAC CCTGTGAAGA GCGTGCGTGT GCTGCGGCCC GAGCCCCAGA CAGCCGTGGG GCCCTCGCAC CCCGCCTGGG
  I  L  H   P  V  K   S  V  R  V   L  R  P   E  P  Q   T  A  V  G   P  S  H   P  A  W

      1130       1140       1150       1160       1170       1180       1190       1200
TGGCTGCGCC CACAGCACCT GCCACTGAGA GCCTGGAGAC GAAGGAGGGC AGCGCAGGCC CACACCCGCT GGATGTGGAC
 V  A  A  P   T  A  P   A  T  E   S  L  E  T   K  E  G   S  A  G   P  H  P  L   D  V  D

      1210       1220       1230       1240       1250       1260       1270       1280
TATGGCGGCT CCGAGCGCAG GTGCCCACCG CCTCCGTATC CAAAGCACTT GCTGCTGCCC AGTAAGTCTG AGCAGTACAG
 Y  G  G   S  E  R  R   C  P  P   P  P  Y   P  K  H  L   L  L  P   S  K  S   E  Q  Y  S

      1290       1300       1310       1320       1330       1340       1350       1360
CGTGGACCTG GACAGCCTGT GCACCAGTGT GCAGCAGAGT CTGCGAGGGG GCACTGATCT AGACGGGAGT GACAAGAGCC
  V  D  L   D  S  L   C  T  S  V   Q  Q  S   L  R  G   G  T  D  L   D  G  S   D  K  S

      1370       1380       1390       1400       1410       1420       1430       1440
ACAAAGGTGC GAAGGGAGAC AAAGCTGGCA GAGACAAAAA GCAGATTCAG ACCTCCCCGG TGCCTGTCCG CAAGAATAGC
 H  K  G  A   K  G  D   K  A  G   R  D  K  K   Q  I  Q   T  S  P   V  P  V  R   K  N  S

      1450       1460       1470       1480       1490       1500       1510       1520
AGAGATGAAG AGAAGAGAGA GTCTCGCATC AAGAGTTACT CCCCTTATGC CTTCAAATTC TTCATGGAGC AACACGTGGA
 R  D  E   E  K  R  E   S  R  I   K  S  Y   S  P  Y  A   F  K  F   F  M  E   Q  H  V  E

      1530       1540       1550       1560       1570       1580       1590       1600
GAATGTCATC AAAACCTACC AGCAGAAGGT CAGCCGGAGG CTACAGCTGG AGCAGGAAAT GGCCAAAGCT GGGCTCTGTG
  N  V  I   K  T  Y   Q  Q  K  V   S  R  R   L  Q  L   E  Q  E  M   A  K  A   G  L  C

      1610       1620       1630       1640       1650       1660       1670       1680
AGGCCGAGCA GGAGCAGATG AGGAAGATCC TCTACCAGAA GGAGTCTAAC TACAACCGGC TGAAGAGGGC CAAGATGGAC
 E  A  E  Q   E  Q  M   R  K  I   L  Y  Q  K   E  S  N   Y  N  R   L  K  R  A   K  M  D

      1690       1700       1710       1720       1730       1740       1750       1760
AAGTCCATGT TTGTGAAAAT CAAGACTCTA GGCATCGGTG CCTTTGGGGA AGTGTGCCTC GCTTGTAAGC TGGACACTCA
 K  S  M   F  V  K  I   K  T  L   G  I  G   A  F  G  E   V  C  L   A  C  K   L  D  T  H

      1770       1780       1790       1800       1810       1820       1830       1840
CGCTCTGTAC GCCATGAAGA CTCTCAGGAA GAAGGATGTC CTGAACCGGA ATCAAGTGGC CCATGTCAAG GCTGAGAGGG
  A  L  Y   A  M  K   T  L  R  K   K  D  V   L  N  R   N  Q  V  A   H  V  K   A  E  R

      1850       1860       1870       1880       1890       1900       1910       1920
ACATCCTGGC TGAAGCAGAC AATGAGTGGG TGGTCAAACT CTACTACTCC TTCCAGGACA AGGACAGCCT GTACTTTGTG
 D  I  L  A   E  A  D   N  E  W   V  V  K  L   Y  Y  S   F  Q  D   K  D  S  L   Y  F  V

      1930       1940       1950       1960       1970       1980       1990       2000
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FIG. 14 (cont.)

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       970        980        990       1000       1010       1020       1030       1040
AGGACCAACT CCTTCAACAA CCCACAACCT GAGCCCTCAC TGCCCGCCCC CAACACGGTC ACCGCCGTGA CGGCCGCACA
 R  T  N   S  F  N  N   P  Q  P   E  P  S   L  P  A  P   N  T  V   T  A  V   T  A  A  H

      1050       1060       1070       1080       1090       1100       1110       1120
CATCCTTCAC CCTGTGAAGA GCGTGCGTGT GCTGCGGCCC GAGCCCCAGA CAGCCGTGGG GCCCTCGCAC CCCGCCTGGG
  I  L  H   P  V  K   S  V  R  V   L  R  P   E  P  Q   T  A  V  G   P  S  H   P  A  W

      1130       1140       1150       1160       1170       1180       1190       1200
TGGCTGCGCC CACAGCACCT GCCACTGAGA GCCTGGAGAC GAAGGAGGGC AGCGCAGGCC CACACCCGCT GGATGTGGAC
V  A  A  P   T  A  P   A  T  E   S  L  E  T   K  E  G   S  A  G   P  H  P  L   D  V  D

      1210       1220       1230       1240       1250       1260       1270       1280
TATGGCGGCT CCGAGCGCAG GTGCCCACCG CCTCCGTATC CAAAGCACTT GCTGCTGCCC AGTAAGTCTG AGCAGTACAG
 Y  G  G   S  E  R  R   C  P  P   P  P  Y   P  K  H  L   L  L  P   S  K  S   E  Q  Y  S

      1290       1300       1310       1320       1330       1340       1350       1360
CGTGGACCTG GACAGCCTGT GCACCAGTGT GCAGCAGAGT CTGCGAGGGG GCACTGATCT AGACGGGAGT GACAAGAGCC
  V  D  L   D  S  L   C  T  S  V   Q  Q  S   L  R  G   G  T  D  L   D  G  S   D  K  S

      1370       1380       1390       1400       1410       1420       1430       1440
ACAAAGGTGC GAAGGGAGAC AAAGCTGGCA GAGACAAAAA GCAGATTCAG ACCTCCCCGG TGCCTGTCCG CAAGAATAGC
H  K  G  A   K  G  D   K  A  G   R  D  K  K   Q  I  Q   T  S  P   V  P  V  R   K  N  S

      1450       1460       1470       1480       1490       1500       1510       1520
AGAGATGAAG AGAAGAGAGA GTCTCGCATC AAGAGTTACT CCCCTTATGC CTTCAAATTC TTCATGGAGC AACACGTGGA
 R  D  E   E  K  R  E   S  R  I   K  S  Y   S  P  Y  A   F  K  F   F  M  E   Q  H  V  E

      1530       1540       1550       1560       1570       1580       1590       1600
GAATGTCATC AAAACCTACC AGCAGAAGGT CAGCCGGAGG CTACAGCTGG AGCAGGAAAT GGCCAAAGCT GGGCTCTGTG
  N  V  I   K  T  Y   Q  Q  K  V   S  R  R   L  Q  L   E  Q  E  M   A  K  A   G  L  C

      1610       1620       1630       1640       1650       1660       1670       1680
AGGCCGAGCA GGAGCAGATG AGGAAGATCC TCTACCAGAA GGAGTCTAAC TACAACCGGC TGAAGAGGGC CAAGATGGAC
E  A  E  Q   E  Q  M   R  K  I   L  Y  Q  K   E  S  N   Y  N  R   L  K  R  A   K  M  D

      1690       1700       1710       1720       1730       1740       1750       1760
AAGTCCATGT TTGTGAAAAT CAAGACTCTA GGCATCGGTG CCTTTGGGGA AGTGTGCCTC GCTTGTAAGC TGGACACTCA
 K  S  M   F  V  K  I   K  T  L   G  I  G   A  F  G  E   V  C  L   A  C  K   L  D  T  H

      1770       1780       1790       1800       1810       1820       1830       1840
CGCTCTGTAC GCCATGAAGA CTCTCAGGAA GAAGGATGTC CTGAACCGGA ATCAAGTGGC CCATGTCAAG GCTGAGAGGG
  A  L  Y   A  M  K   T  L  R  K   K  D  V   L  N  R   N  Q  V  A   H  V  K   A  E  R

      1850       1860       1870       1880       1890       1900       1910       1920
ACATCCTGGC TGAAGCAGAC AATGAGTGGG TGGTCAAACT CTACTACTCC TTCCAGGACA AGGACAGCCT GTACTTTGTG
D  I  L  A   E  A  D   N  E  W   V  V  K  L   Y  Y  S   F  Q  D   K  D  S  L   Y  F  V

      1930       1940       1950       1960       1970       1980       1990       2000
```

FIG. 14 (cont.)

```
      2970       2980       2990       3000       3010       3020       3030       3040
         *          *          *          *          *          *          *          *
CTCGAGGAAA CCCAAAATGA GATTTCTTTT CAGAAGACAA ACTCAAGCTT AGGAATCCTT CATTTTTAGT TCTGGTAAAT

      3050       3060       3070       3080       3090       3100       3110       3120
         *          *          *          *          *          *          *          *
GGGCAACAGG AAGAGTCAAC ATGATTTCAA ATTAGCCCTC TGAGGACCTT CACTGCATTA AAACAGTATT TTTTAAAAAA

      3130       3140       3150
         *          *          *
TTAGTACAGT ATGGAAAGAG CACTTATTTT GGGGG
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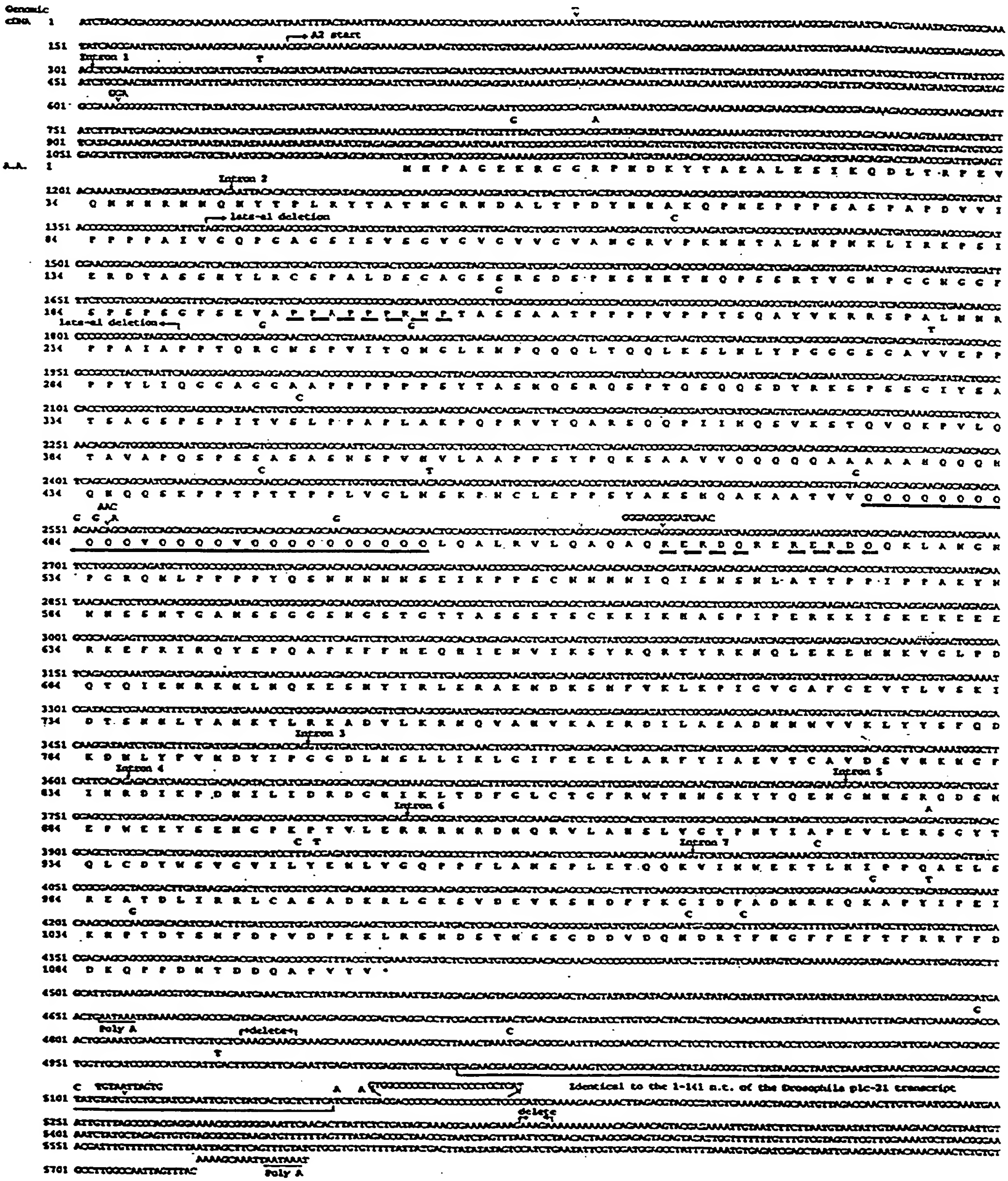
FIG. 14 (cont.)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A.A.
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Intron 1
Intron 2
lats-a1 deletion
Intron 3
Intron 4
Intron 5
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Poly A
delete
Identical to the 1-141 n.t. of the Drosophila plc-21 transcript

FIG. 15

SEQUENCE LISTING

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      DISORDERS WITH LATS PROTEINS, DERIVATIVES AND
      FRAGMENTS, AND LATS KNOCK-OUT ANIMAL MODELS

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acagtcctgg ggacttcctt gaaggatcat tttcactttt gctcagaaga aagctctgga 180

tctatcaaat aaagaagtcc ttcgtgtggg ctacatatat agatgttttc atg aag     236
                                                       Met Lys
                                                         1

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Arg Ser Glu Lys Pro Glu Gly Tyr Arg Gln Met Arg Pro Lys Thr Phe
          5                  10                  15

cct gcc agt aac tat act gtc agt agc cgg caa atg tta caa gaa att    332
Pro Ala Ser Asn Tyr Thr Val Ser Ser Arg Gln Met Leu Gln Glu Ile
     20                  25                  30

cgg gaa tcc ctt agg aat tta tct aaa cca tct gat gct gct aag gct    380
Arg Glu Ser Leu Arg Asn Leu Ser Lys Pro Ser Asp Ala Ala Lys Ala
 35                  40                  45                  50

gag cat aac atg agt aaa atg tca acc gaa gat cct cga caa gtc aga    428
Glu His Asn Met Ser Lys Met Ser Thr Glu Asp Pro Arg Gln Val Arg
                 55                  60                  65

aat cca ccc aaa ttt ggg acg cat cat aaa gcc ttg cag gaa att cga    476
Asn Pro Pro Lys Phe Gly Thr His His Lys Ala Leu Gln Glu Ile Arg
             70                  75                  80
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Asn Ser Leu Leu Pro Phe Ala Asn Glu Thr Asn Ser Ser Arg Ser Thr
         85                  90                  95

tca gaa gtt aat cca caa atg ctt caa gac ttg caa gct gct gga ttt    572
Ser Glu Val Asn Pro Gln Met Leu Gln Asp Leu Gln Ala Ala Gly Phe
    100                 105                 110

gat gag gat atg gtt ata caa gct ctt cag aaa act aac aac aga agt    620
Asp Glu Asp Met Val Ile Gln Ala Leu Gln Lys Thr Asn Asn Arg Ser
115                 120                 125                 130

ata gaa gca gca att gaa ttc att agt aaa atg agt tac caa gat cct    668
Ile Glu Ala Ala Ile Glu Phe Ile Ser Lys Met Ser Tyr Gln Asp Pro
                135                 140                 145

cga cga gag cag atg gct gca gca gct gcc aga cct att aat gcc agc    716
Arg Arg Glu Gln Met Ala Ala Ala Ala Ala Arg Pro Ile Asn Ala Ser
            150                 155                 160

atg aaa cca ggg aat gtg cag caa tca gtt aac cgc aaa cag agc tgg    764
Met Lys Pro Gly Asn Val Gln Gln Ser Val Asn Arg Lys Gln Ser Trp
        165                 170                 175

aaa ggt tct aaa gaa tcc tta gtt cct cag agg cat ggc ccg cca cta    812
Lys Gly Ser Lys Glu Ser Leu Val Pro Gln Arg His Gly Pro Pro Leu
    180                 185                 190

gga gaa agt gtg gcc tat cat tct gag agt ccc aac tca cag aca gat    860
Gly Glu Ser Val Ala Tyr His Ser Glu Ser Pro Asn Ser Gln Thr Asp
195                 200                 205                 210

gta gga aga cct ttg tct gga tct ggt ata tca gca ttt gtt caa gct    908
Val Gly Arg Pro Leu Ser Gly Ser Gly Ile Ser Ala Phe Val Gln Ala
                215                 220                 225

cac cct agc aac gga cag aga gtg aac ccc cca cca cca cct caa gta    956
His Pro Ser Asn Gly Gln Arg Val Asn Pro Pro Pro Pro Pro Gln Val
            230                 235                 240

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Arg Ser Val Thr Pro Pro Pro Pro Pro Arg Gly Gln Thr Pro Pro Pro
        245                 250                 255

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Arg Gly Thr Thr Pro Pro Pro Pro Ser Trp Glu Pro Asn Ser Gln Thr
    260                 265                 270

aag cgc tat tct gga aac atg gaa tac gta atc tcc cga atc tct cct    1100
Lys Arg Tyr Ser Gly Asn Met Glu Tyr Val Ile Ser Arg Ile Ser Pro
275                 280                 285                 290

gtc cca cct ggg gca tgg caa gag ggc tat cct cca cca cct ctc aac    1148
Val Pro Pro Gly Ala Trp Gln Glu Gly Tyr Pro Pro Pro Pro Leu Asn
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Val Pro Val Gly Arg Gln Pro Ile Ile Met Gln Ser Ser Ser Lys Phe
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Asn Phe Pro Ser Gly Arg Pro Gly Met Gln Asn Gly Thr Gly Gln Thr
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Asp Phe Met Ile His Gln Asn Val Val Pro Ala Gly Thr Val Asn Arg
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Gln Pro Pro Pro Pro Tyr Pro Leu Thr Ala Ala Asn Gly Gln Ser Pro
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Ser Ala Leu Gln Thr Gly Gly Ser Ala Ala Pro Ser Ser Tyr Thr Asn
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His Arg Lys Lys Gln Leu Glu Asn Glu Met Met Arg Val Gly Leu Ser
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695 700 705

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Lys Ile Lys Thr Leu Gly Ile Gly Ala Phe Gly Glu Val Cys Leu Ala
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Asp Ile Leu Ala Glu Ala Asp Asn Glu Trp Val Val Arg Leu Tyr Tyr
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Ser Phe Gln Asp Lys Asp Asn Leu Tyr Phe Val Met Asp Tyr Ile Pro
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ggg ggt gat atg atg agc cta tta att aga atg ggc atc ttt cca gaa   2636
Gly Gly Asp Met Met Ser Leu Leu Ile Arg Met Gly Ile Phe Pro Glu
            790                 795                 800

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Ser Leu Ala Arg Phe Tyr Ile Ala Glu Leu Thr Cys Ala Val Glu Ser
        805                 810                 815

gtt cat aaa atg ggt ttt att cat aga gat att aaa cct gat aat att   2732
Val His Lys Met Gly Phe Ile His Arg Asp Ile Lys Pro Asp Asn Ile
    820                 825                 830

ttg att gat cgt gat ggt cat att aaa ttg act gac ttt ggc ctc tgc   2780
Leu Ile Asp Arg Asp Gly His Ile Lys Leu Thr Asp Phe Gly Leu Cys
835                 840                 845                 850

act ggc ttc aga tgg aca cac gat tct aag tac tat cag agt ggt gac   2828
Thr Gly Phe Arg Trp Thr His Asp Ser Lys Tyr Tyr Gln Ser Gly Asp
                855                 860                 865

cat cca cgg caa gat agc atg gat ttc agt aat gaa tgg ggg gat ccc   2876
His Pro Arg Gln Asp Ser Met Asp Phe Ser Asn Glu Trp Gly Asp Pro
            870                 875                 880

tca agc tgt cga tgt gga gac aga ctg aag cca tta gag cgg aga gct   2924
Ser Ser Cys Arg Cys Gly Asp Arg Leu Lys Pro Leu Glu Arg Arg Ala
        885                 890                 895

gca cgc cag cac cag cga tgt cta gca cat tct ttg gtt ggg act ccc   2972
Ala Arg Gln His Gln Arg Cys Leu Ala His Ser Leu Val Gly Thr Pro
    900                 905                 910

aat tat att gca cct gaa gtg ttg cta cga aca gga tac aca cag ttg   3020
Asn Tyr Ile Ala Pro Glu Val Leu Leu Arg Thr Gly Tyr Thr Gln Leu
915                 920                 925                 930

tgt gat tgg tgg agt gtt ggt gtt att ctt ttt gaa atg ttg gtg gga   3068
Cys Asp Trp Trp Ser Val Gly Val Ile Leu Phe Glu Met Leu Val Gly
                935                 940                 945

caa cct cct ttc ttg gca caa aca cca tta gaa aca caa atg aag gtt   3116
Gln Pro Pro Phe Leu Ala Gln Thr Pro Leu Glu Thr Gln Met Lys Val
            950                 955                 960

atc aac tgg caa aca tct ctt cac att cca cca caa gct aaa ctc agt   3164
Ile Asn Trp Gln Thr Ser Leu His Ile Pro Pro Gln Ala Lys Leu Ser
        965                 970                 975
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980 985 990

cgc tta ggc aag aat ggt gct gat gaa ata aaa gct cat cca ttt ttt 3260
Arg Leu Gly Lys Asn Gly Ala Asp Glu Ile Lys Ala His Pro Phe Phe
995 1000 1005 1010

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Lys Thr Ile Asp Phe Ser Ser Asp Leu Arg Gln Gln Ser Ala Ser Tyr
1015 1020 1025

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Ile Pro Lys Ile Thr His Pro Thr Asp Thr Ser Asn Phe Asp Pro Val
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Val Arg Asn Pro Pro Lys Phe Gly Thr His His Lys Ala Leu Gln Glu
65 70 75 80

Ile Arg Asn Ser Leu Leu Pro Phe Ala Asn Glu Thr Asn Ser Ser Arg
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Ser Thr Ser Glu Val Asn Pro Gln Met Leu Gln Asp Leu Gln Ala Ala
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Gly Phe Asp Glu Asp Met Val Ile Gln Ala Leu Gln Lys Thr Asn Asn
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Arg Ser Ile Glu Ala Ala Ile Glu Phe Ile Ser Lys Met Ser Tyr Gln
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Leu Ser Gln Asp Ala Gln Asp Gln Met Arg Lys Met Leu Cys Gln Lys
675 680 685

Glu Ser Asn Tyr Ile Arg Leu Lys Arg Ala Lys Met Asp Lys Ser Met
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Leu Ala Arg Lys Val Asp Thr Lys Ala Leu Tyr Ala Thr Lys Thr Leu
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Arg Lys Lys Asp Val Leu Leu Arg Asn Gln Val Ala His Val Lys Ala
740 745 750

Glu Arg Asp Ile Leu Ala Glu Ala Asp Asn Glu Trp Val Val Arg Leu
755 760 765

Tyr Tyr Ser Phe Gln Asp Lys Asp Asn Leu Tyr Phe Val Met Asp Tyr
770 775 780

Ile Pro Gly Gly Asp Met Met Ser Leu Leu Ile Arg Met Gly Ile Phe
785 790 795 800

Pro Glu Ser Leu Ala Arg Phe Tyr Ile Ala Glu Leu Thr Cys Ala Val
805 810 815

Glu Ser Val His Lys Met Gly Phe Ile His Arg Asp Ile Lys Pro Asp
820 825 830

Asn Ile Leu Ile Asp Arg Asp Gly His Ile Lys Leu Thr Asp Phe Gly
835 840 845

Leu Cys Thr Gly Phe Arg Trp Thr His Asp Ser Lys Tyr Tyr Gln Ser
850 855 860

Gly Asp His Pro Arg Gln Asp Ser Met Asp Phe Ser Asn Glu Trp Gly
865 870 875 880

Asp Pro Ser Ser Cys Arg Cys Gly Asp Arg Leu Lys Pro Leu Glu Arg
885 890 895

Arg Ala Ala Arg Gln His Gln Arg Cys Leu Ala His Ser Leu Val Gly
900 905 910

Thr Pro Asn Tyr Ile Ala Pro Glu Val Leu Leu Arg Thr Gly Tyr Thr
915 920 925

Gln Leu Cys Asp Trp Trp Ser Val Gly Val Ile Leu Phe Glu Met Leu
930 935 940

Val Gly Gln Pro Pro Phe Leu Ala Gln Thr Pro Leu Glu Thr Gln Met
945 950 955 960

Lys Val Ile Asn Trp Gln Thr Ser Leu His Ile Pro Pro Gln Ala Lys
965 970 975

Leu Ser Pro Glu Ala Ser Asp Leu Ile Ile Lys Leu Cys Arg Gly Pro
980 985 990

Glu Asp Arg Leu Gly Lys Asn Gly Ala Asp Glu Ile Lys Ala His Pro
995 1000 1005

Phe Phe Lys Thr Ile Asp Phe Ser Ser Asp Leu Arg Gln Gln Ser Ala
1010 1015 1020

Ser Tyr Ile Pro Lys Ile Thr His Pro Thr Asp Thr Ser Asn Phe Asp
025 1030 1035 1040

Pro Val Asp Pro Asp Lys Leu Trp Ser Asp Asp Asn Glu Glu Glu Asn
1045 1050 1055

Val Asn Asp Thr Leu Asn Gly Trp Tyr Lys Asn Gly Lys His Pro Glu
1060 1065 1070

His Ala Phe Tyr Glu Phe Thr Phe Arg Arg Phe Phe Asp Asp Asn Gly
1075 1080 1085

Tyr Pro Tyr Asn Tyr Pro Lys Pro Ile Glu Tyr Glu Tyr Ile Asn Ser
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Gln Gly Ser Glu Gln Gln Ser Asp Glu Asp Asp Gln Asn Thr Gly Ser
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tct cta gtt cct cag aga cac ggc cca tct cta gga gaa aat gtg gtt      96
Ser Leu Val Pro Gln Arg His Gly Pro Ser Leu Gly Glu Asn Val Val
             20                  25                  30

tat cgt tct gaa agc ccc aac tca cag gcg gat gta gga aga cct ctg     144
Tyr Arg Ser Glu Ser Pro Asn Ser Gln Ala Asp Val Gly Arg Pro Leu
         35                  40                  45

tct gga tcc ggc att gca gca ttt gct caa gct cac cca agc aat gga     192
Ser Gly Ser Gly Ile Ala Ala Phe Ala Gln Ala His Pro Ser Asn Gly
     50                  55                  60

cag aga gtg aac ccc cca cca cca cct caa gtt agg agt gtt act cct     240
Gln Arg Val Asn Pro Pro Pro Pro Pro Gln Val Arg Ser Val Thr Pro
 65                  70                  75                  80

cca cca cct ccg aga ggc cag acc cca cct ccc cga ggc acc act ccc     288
Pro Pro Pro Pro Arg Gly Gln Thr Pro Pro Pro Arg Gly Thr Thr Pro
                 85                  90                  95

cct ccc ccc tca tgg gaa cca agc tct cag aca aag cgc tac tct ggg     336
Pro Pro Pro Ser Trp Glu Pro Ser Ser Gln Thr Lys Arg Tyr Ser Gly
            100                 105                 110

aac atg gag tac gta atc tcc cga atc tcc cct gtt cca cct ggg gcg     384
Asn Met Glu Tyr Val Ile Ser Arg Ile Ser Pro Val Pro Pro Gly Ala
        115                 120                 125

tgg cag gag ggg tac cct cca cca cct ctt acc act tct ccc atg aat     432
Trp Gln Glu Gly Tyr Pro Pro Pro Pro Leu Thr Thr Ser Pro Met Asn
    130                 135                 140

ccc cct agc cag gct cag agg gcc att agt tct gtt cca gtt ggt aga     480
Pro Pro Ser Gln Ala Gln Arg Ala Ile Ser Ser Val Pro Val Gly Arg
145                 150                 155                 160

caa ccc atc atc atg cag agt act agc aaa ttt aac ttt aca cca ggg     528
Gln Pro Ile Ile Met Gln Ser Thr Ser Lys Phe Asn Phe Thr Pro Gly
                165                 170                 175

cga cct gga gtt cag aat ggt ggt ggt cag tct gat ttt atc gtg cac     576
Arg Pro Gly Val Gln Asn Gly Gly Gly Gln Ser Asp Phe Ile Val His
            180                 185                 190

caa aat gtc ccc act ggt tct gtg act cgg cag cca cca cct cca tat     624
Gln Asn Val Pro Thr Gly Ser Val Thr Arg Gln Pro Pro Pro Pro Tyr
        195                 200                 205

cct ctg acc cca gct aat gga caa agc ccc tct gct tta caa aca ggg     672
Pro Leu Thr Pro Ala Asn Gly Gln Ser Pro Ser Ala Leu Gln Thr Gly
    210                 215                 220

gct tct gct gct cca cca tca ttc gcc aat gga aac gtt cct cag tcg     720
Ala Ser Ala Ala Pro Pro Ser Phe Ala Asn Gly Asn Val Pro Gln Ser
225                 230                 235                 240
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atg atg gtg ccc aac agg aac agt cat aac atg gag ctt tat aat att 768
Met Met Val Pro Asn Arg Asn Ser His Asn Met Glu Leu Tyr Asn Ile
245 250 255

aat gtc cct gga ctg caa aca gcc tgg ccc cag tcg tct tct gct cct 816
Asn Val Pro Gly Leu Gln Thr Ala Trp Pro Gln Ser Ser Ser Ala Pro
260 265 270

gcg cag tca tcc cca agc ggt ggg cat gaa att cct aca tgg caa cct 864
Ala Gln Ser Ser Pro Ser Gly Gly His Glu Ile Pro Thr Trp Gln Pro
275 280 285

aac ata cca gtg agg tca aat tct ttt aat aac cca tta gga agt aga 912
Asn Ile Pro Val Arg Ser Asn Ser Phe Asn Asn Pro Leu Gly Ser Arg
290 295 300

gca agt cac tct gct aat tct cag cct tct gcc act aca gtc act gcc 960
Ala Ser His Ser Ala Asn Ser Gln Pro Ser Ala Thr Thr Val Thr Ala
305 310 315 320

atc aca ccc gct cct att caa cag ccc gtg aaa agc atg cgc gtc ctg 1008
Ile Thr Pro Ala Pro Ile Gln Gln Pro Val Lys Ser Met Arg Val Leu
325 330 335

aaa cca gag ctg cag act gct tta gcc cca acc cat cct tct tgg atg 1056
Lys Pro Glu Leu Gln Thr Ala Leu Ala Pro Thr His Pro Ser Trp Met
340 345 350

cca cag cca gtt cag act gtt cag cct acc cct ttt tct gag ggt aca 1104
Pro Gln Pro Val Gln Thr Val Gln Pro Thr Pro Phe Ser Glu Gly Thr
355 360 365

gct tca agt gtg cct gtc atc cca cct gtt gct gaa gct cca agc tat 1152
Ala Ser Ser Val Pro Val Ile Pro Pro Val Ala Glu Ala Pro Ser Tyr
370 375 380

caa ggt cca cca ccg cct tat cca aaa cat ctg cta cac caa aac cca 1200
Gln Gly Pro Pro Pro Pro Tyr Pro Lys His Leu Leu His Gln Asn Pro
385 390 395 400

tct gtc cct cca tat gag tca gta agt aag ccc tgc aaa gat gaa cag 1248
Ser Val Pro Pro Tyr Glu Ser Val Ser Lys Pro Cys Lys Asp Glu Gln
405 410 415

cct agc tta ccc aag gaa gat gat agt gag aag agt gcg gac agt ggt 1296
Pro Ser Leu Pro Lys Glu Asp Asp Ser Glu Lys Ser Ala Asp Ser Gly
420 425 430

gac tct ggg gat aaa gaa aag aaa cag att aca act tca cct atc act 1344
Asp Ser Gly Asp Lys Glu Lys Lys Gln Ile Thr Thr Ser Pro Ile Thr
435 440 445

gtt cgg aaa aac aag aaa gat gaa gaa cga aga gag tct cgg att cag 1392
Val Arg Lys Asn Lys Lys Asp Glu Glu Arg Arg Glu Ser Arg Ile Gln
450 455 460

agt tac tcc cca cag gcc ttt aag ttc ttc atg gag cag cac gta gag 1440
Ser Tyr Ser Pro Gln Ala Phe Lys Phe Phe Met Glu Gln His Val Glu
465 470 475 480

aac gtc ctg aag tct cat cag cag cgt ctg cat cgg aag aag cag cta 1488
Asn Val Leu Lys Ser His Gln Gln Arg Leu His Arg Lys Lys Gln Leu
485 490 495

gaa aat gaa atg atg cgg gtt gga tta tct caa gat gcc cag gat caa 1536
Glu Asn Glu Met Met Arg Val Gly Leu Ser Gln Asp Ala Gln Asp Gln
500 505 510

atg aga aag atg ctt tgc cag aaa gag tct aac tat att cgt ctt aaa 1584
Met Arg Lys Met Leu Cys Gln Lys Glu Ser Asn Tyr Ile Arg Leu Lys
515 520 525

agg gct aaa atg gac aag tct atg ttt gta aag ata aag aca tta gga 1632
Arg Ala Lys Met Asp Lys Ser Met Phe Val Lys Ile Lys Thr Leu Gly
530 535 540

ata gga gcg ttt ggt gaa gtc tgt cta gca aga aaa gtc gat act aaa 1680
Ile Gly Ala Phe Gly Glu Val Cys Leu Ala Arg Lys Val Asp Thr Lys
545 550 555 560

gct ttg tat gca aca aag act ctt cga aag aaa gac gtt ctg ctc cga 1728
Ala Leu Tyr Ala Thr Lys Thr Leu Arg Lys Lys Asp Val Leu Leu Arg
565 570 575

aat cag gtg gct cat gtg aaa gcg gag agg gat atc cta gca gaa gcc 1776
Asn Gln Val Ala His Val Lys Ala Glu Arg Asp Ile Leu Ala Glu Ala
580 585 590

gac aat gag tgg gtg gtc cgc ctg tac tac tct ttc cag gac aag gac 1824
Asp Asn Glu Trp Val Val Arg Leu Tyr Tyr Ser Phe Gln Asp Lys Asp
595 600 605

aac ttg tac ttt gtg atg gac tac att cct ggg ggg gat atg atg agc 1872
Asn Leu Tyr Phe Val Met Asp Tyr Ile Pro Gly Gly Asp Met Met Ser
610 615 620

cta tta att aga atg ggc atc ttt cct gaa aat ctg gca cga ttc tac 1920
Leu Leu Ile Arg Met Gly Ile Phe Pro Glu Asn Leu Ala Arg Phe Tyr
625 630 635 640

ata gca gaa ctt acc tgt gca gtt gaa agt gtt cat aaa atg ggt ttt 1968
Ile Ala Glu Leu Thr Cys Ala Val Glu Ser Val His Lys Met Gly Phe
645 650 655

att cat aga gat att aaa cct gat aac att ttg att gac cgt gat ggc 2016
Ile His Arg Asp Ile Lys Pro Asp Asn Ile Leu Ile Asp Arg Asp Gly
660 665 670

cat att aaa ttg act gac ttt ggc ttg tgc act ggc ttc aga tgg aca 2064
His Ile Lys Leu Thr Asp Phe Gly Leu Cys Thr Gly Phe Arg Trp Thr
675 680 685

cat gac tcc aag tac tac cag agt ggg gat cac cca cgg caa gat agc 2112
His Asp Ser Lys Tyr Tyr Gln Ser Gly Asp His Pro Arg Gln Asp Ser
690 695 700

atg gat ttc agt aac gaa tgg gga gat cct tcc aat tgt cgg tgt ggg 2160
Met Asp Phe Ser Asn Glu Trp Gly Asp Pro Ser Asn Cys Arg Cys Gly
705 710 715 720

gac aga ctg aag cca ctg gag cgg aga gct gct cgc cag cac cag cga 2208
Asp Arg Leu Lys Pro Leu Glu Arg Arg Ala Ala Arg Gln His Gln Arg
725 730 735

tgt cta gcc cat tct ctg gtt ggg act ccc aat tat att gca cct gaa 2256
Cys Leu Ala His Ser Leu Val Gly Thr Pro Asn Tyr Ile Ala Pro Glu
740 745 750

gtg cta ctg cga aca gga tat aca cag ctg tgt gac tgg tgg agt gtt 2304
Val Leu Leu Arg Thr Gly Tyr Thr Gln Leu Cys Asp Trp Trp Ser Val
755 760 765

ggt gtt att ctt tgt gaa atg ttg gtg gga caa cct cct ttc ttg gca 2352
Gly Val Ile Leu Cys Glu Met Leu Val Gly Gln Pro Pro Phe Leu Ala
770 775 780

caa acc cca tta gaa aca caa atg aag gtt atc atc tgg caa act tct 2400
Gln Thr Pro Leu Glu Thr Gln Met Lys Val Ile Ile Trp Gln Thr Ser
785 790 795 800

cta cac atc cct cct caa gct aag ctg agt cct gaa gcc tct gac ctc 2448
Leu His Ile Pro Pro Gln Ala Lys Leu Ser Pro Glu Ala Ser Asp Leu
805 810 815

att atc aaa ctg tgt cga gga cca gaa gac cgc ctc ggc aag aac ggt 2496
Ile Ile Lys Leu Cys Arg Gly Pro Glu Asp Arg Leu Gly Lys Asn Gly
820 825 830

gct gat gag ata aag gct cat cca ttt ttt aag acc atc gat ttc tct 2544
Ala Asp Glu Ile Lys Ala His Pro Phe Phe Lys Thr Ile Asp Phe Ser
835 840 845

agt gat ctg aga cag cag tct gct tca tac atc cct aaa atc acg cat 2592
Ser Asp Leu Arg Gln Gln Ser Ala Ser Tyr Ile Pro Lys Ile Thr His
850 855 860

cca aca gat aca tcc aat ttc gac cct gtt gat cct gat aaa ttg tgg 2640
Pro Thr Asp Thr Ser Asn Phe Asp Pro Val Asp Pro Asp Lys Leu Trp
865 870 875 880

agc gat ggc agc gag gag gaa aat atc agt gac act ctg agc gga tgg 2688
Ser Asp Gly Ser Glu Glu Glu Asn Ile Ser Asp Thr Leu Ser Gly Trp
885 890 895

tat aaa aat ggg aag cac ccc gag cac gct ttc tat gag ttc acc ttt 2736
Tyr Lys Asn Gly Lys His Pro Glu His Ala Phe Tyr Glu Phe Thr Phe
900 905 910

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cgg agg ttt ttt gat gac aat ggc tac cca tat aat tat cca aag cct    2784
Arg Arg Phe Phe Asp Asp Asn Gly Tyr Pro Tyr Asn Tyr Pro Lys Pro
        915                 920                 925

att gag tat gaa tac att cat tca cag ggc tca gaa caa cag tct gat    2832
Ile Glu Tyr Glu Tyr Ile His Ser Gln Gly Ser Glu Gln Gln Ser Asp
    930                 935                 940

gaa gat gat caa cac aca agc tcc gat gga aac aac cga gat cta gtg    2880
Glu Asp Asp Gln His Thr Ser Ser Asp Gly Asn Asn Arg Asp Leu Val
945                 950                 955                 960

tat gtt taataaacta ggagatcatt gtaagaattt gcaagaggcc tgaagtgcag     2936
Tyr Val

gggtttttga agttttgaga aaattatgca aatgtgacag agtttgtgtg ctctgtgtac 2996

aatattttat tttcctaagt tatgggaaat tgttttaaaa tgttaattta ttccaccctt 3056

ttaattcagt aatttagaaa aaattgttat aaggaaagta aattatgaac tgagtattat 3116

agtcaattct tggtacttaa agtacttaaa aagagaagcc tggtatcttt tgtatatata 3176

ataaataatt ttaaaatccc aaaaaaaaaa aaaaaaa                           3213


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&lt;212&gt; PRT
&lt;213&gt; Mus musculus

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            20                  25                  30

Tyr Arg Ser Glu Ser Pro Asn Ser Gln Ala Asp Val Gly Arg Pro Leu
        35                  40                  45

Ser Gly Ser Gly Ile Ala Ala Phe Ala Gln Ala His Pro Ser Asn Gly
    50                  55                  60

Gln Arg Val Asn Pro Pro Pro Pro Pro Gln Val Arg Ser Val Thr Pro
65                  70                  75                  80

Pro Pro Pro Pro Arg Gly Gln Thr Pro Pro Pro Arg Gly Thr Thr Pro
                85                  90                  95

Pro Pro Pro Ser Trp Glu Pro Ser Ser Gln Thr Lys Arg Tyr Ser Gly
            100                 105                 110

Asn Met Glu Tyr Val Ile Ser Arg Ile Ser Pro Val Pro Pro Gly Ala
        115                 120                 125

Trp Gln Glu Gly Tyr Pro Pro Pro Pro Leu Thr Thr Ser Pro Met Asn
    130                 135                 140
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Pro Pro Ser Gln Ala Gln Arg Ala Ile Ser Ser Val Pro Val Gly Arg
145                 150                 155                 160

Gln Pro Ile Ile Met Gln Ser Thr Ser Lys Phe Asn Phe Thr Pro Gly
                165                 170                 175

Arg Pro Gly Val Gln Asn Gly Gly Gly Gln Ser Asp Phe Ile Val His
            180                 185                 190

Gln Asn Val Pro Thr Gly Ser Val Thr Arg Gln Pro Pro Pro Pro Tyr
        195                 200                 205

Pro Leu Thr Pro Ala Asn Gly Gln Ser Pro Ser Ala Leu Gln Thr Gly
    210                 215                 220

Ala Ser Ala Ala Pro Pro Ser Phe Ala Asn Gly Asn Val Pro Gln Ser
225                 230                 235                 240

Met Met Val Pro Asn Arg Asn Ser His Asn Met Glu Leu Tyr Asn Ile
                245                 250                 255

Asn Val Pro Gly Leu Gln Thr Ala Trp Pro Gln Ser Ser Ser Ala Pro
            260                 265                 270

Ala Gln Ser Ser Pro Ser Gly Gly His Glu Ile Pro Thr Trp Gln Pro
        275                 280                 285

Asn Ile Pro Val Arg Ser Asn Ser Phe Asn Asn Pro Leu Gly Ser Arg
    290                 295                 300

Ala Ser His Ser Ala Asn Ser Gln Pro Ser Ala Thr Thr Val Thr Ala
305                 310                 315                 320

Ile Thr Pro Ala Pro Ile Gln Gln Pro Val Lys Ser Met Arg Val Leu
                325                 330                 335

Lys Pro Glu Leu Gln Thr Ala Leu Ala Pro Thr His Pro Ser Trp Met
            340                 345                 350

Pro Gln Pro Val Gln Thr Val Gln Pro Thr Pro Phe Ser Glu Gly Thr
        355                 360                 365

Ala Ser Ser Val Pro Val Ile Pro Pro Val Ala Glu Ala Pro Ser Tyr
    370                 375                 380

Gln Gly Pro Pro Pro Pro Tyr Pro Lys His Leu Leu His Gln Asn Pro
385                 390                 395                 400

Ser Val Pro Pro Tyr Glu Ser Val Ser Lys Pro Cys Lys Asp Glu Gln
                405                 410                 415

Pro Ser Leu Pro Lys Glu Asp Asp Ser Glu Lys Ser Ala Asp Ser Gly
            420                 425                 430

Asp Ser Gly Asp Lys Glu Lys Lys Gln Ile Thr Thr Ser Pro Ile Thr
        435                 440                 445
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Val Arg Lys Asn Lys Lys Asp Glu Glu Arg Arg Glu Ser Arg Ile Gln
450 455 460

Ser Tyr Ser Pro Gln Ala Phe Lys Phe Phe Met Glu Gln His Val Glu
465 470 475 480

Asn Val Leu Lys Ser His Gln Gln Arg Leu His Arg Lys Lys Gln Leu
485 490 495

Glu Asn Glu Met Met Arg Val Gly Leu Ser Gln Asp Ala Gln Asp Gln
500 505 510

Met Arg Lys Met Leu Cys Gln Lys Glu Ser Asn Tyr Ile Arg Leu Lys
515 520 525

Arg Ala Lys Met Asp Lys Ser Met Phe Val Lys Ile Lys Thr Leu Gly
530 535 540

Ile Gly Ala Phe Gly Glu Val Cys Leu Ala Arg Lys Val Asp Thr Lys
545 550 555 560

Ala Leu Tyr Ala Thr Lys Thr Leu Arg Lys Lys Asp Val Leu Leu Arg
565 570 575

Asn Gln Val Ala His Val Lys Ala Glu Arg Asp Ile Leu Ala Glu Ala
580 585 590

Asp Asn Glu Trp Val Val Arg Leu Tyr Tyr Ser Phe Gln Asp Lys Asp
595 600 605

Asn Leu Tyr Phe Val Met Asp Tyr Ile Pro Gly Gly Asp Met Met Ser
610 615 620

Leu Leu Ile Arg Met Gly Ile Phe Pro Glu Asn Leu Ala Arg Phe Tyr
625 630 635 640

Ile Ala Glu Leu Thr Cys Ala Val Glu Ser Val His Lys Met Gly Phe
645 650 655

Ile His Arg Asp Ile Lys Pro Asp Asn Ile Leu Ile Asp Arg Asp Gly
660 665 670

His Ile Lys Leu Thr Asp Phe Gly Leu Cys Thr Gly Phe Arg Trp Thr
675 680 685

His Asp Ser Lys Tyr Tyr Gln Ser Gly Asp His Pro Arg Gln Asp Ser
690 695 700

Met Asp Phe Ser Asn Glu Trp Gly Asp Pro Ser Asn Cys Arg Cys Gly
705 710 715 720

Asp Arg Leu Lys Pro Leu Glu Arg Arg Ala Ala Arg Gln His Gln Arg
725 730 735

Cys Leu Ala His Ser Leu Val Gly Thr Pro Asn Tyr Ile Ala Pro Glu
740 745 750

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Val Leu Leu Arg Thr Gly Tyr Thr Gln Leu Cys Asp Trp Trp Ser Val
        755                 760                 765

Gly Val Ile Leu Cys Glu Met Leu Val Gly Gln Pro Pro Phe Leu Ala
    770                 775                 780

Gln Thr Pro Leu Glu Thr Gln Met Lys Val Ile Ile Trp Gln Thr Ser
785                 790                 795                 800

Leu His Ile Pro Pro Gln Ala Lys Leu Ser Pro Glu Ala Ser Asp Leu
                805                 810                 815

Ile Ile Lys Leu Cys Arg Gly Pro Glu Asp Arg Leu Gly Lys Asn Gly
            820                 825                 830

Ala Asp Glu Ile Lys Ala His Pro Phe Phe Lys Thr Ile Asp Phe Ser
        835                 840                 845

Ser Asp Leu Arg Gln Gln Ser Ala Ser Tyr Ile Pro Lys Ile Thr His
    850                 855                 860

Pro Thr Asp Thr Ser Asn Phe Asp Pro Val Asp Pro Asp Lys Leu Trp
865                 870                 875                 880

Ser Asp Gly Ser Glu Glu Glu Asn Ile Ser Asp Thr Leu Ser Gly Trp
                885                 890                 895

Tyr Lys Asn Gly Lys His Pro Glu His Ala Phe Tyr Glu Phe Thr Phe
            900                 905                 910

Arg Arg Phe Phe Asp Asp Asn Gly Tyr Pro Tyr Asn Tyr Pro Lys Pro
        915                 920                 925

Ile Glu Tyr Glu Tyr Ile His Ser Gln Gly Ser Glu Gln Gln Ser Asp
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Glu Asp Asp Gln His Thr Ser Ser Asp Gly Asn Asn Arg Asp Leu Val
945                 950                 955                 960

Tyr Val


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  1               5                  10                  15
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atc cga tat tcc ctc ctg cct ttt gcc aac gag tca ggc act tcg gca     96
Ile Arg Tyr Ser Leu Leu Pro Phe Ala Asn Glu Ser Gly Thr Ser Ala
             20                  25                  30

gct gca gag gtg aac cgg cag atg ctt cag gag ttg gtg aat gcg gca    144
Ala Ala Glu Val Asn Arg Gln Met Leu Gln Glu Leu Val Asn Ala Ala
         35                  40                  45

tgt gac cag gag atg gct ggc aga gcg ctc acg cag acg ggc agt agg    192
Cys Asp Gln Glu Met Ala Gly Arg Ala Leu Thr Gln Thr Gly Ser Arg
     50                  55                  60

agt atc gaa gct gcc ttg gag tac atc agt aag atg ggc tac ctg gac    240
Ser Ile Glu Ala Ala Leu Glu Tyr Ile Ser Lys Met Gly Tyr Leu Asp
 65                  70                  75                  80

ccc agg aat gag cag att gtg cga gtc atc aag cag acc tcc cca gga    288
Pro Arg Asn Glu Gln Ile Val Arg Val Ile Lys Gln Thr Ser Pro Gly
                 85                  90                  95

aag ggc ctg gcg tcc acc ccg gtg act cgg cgg ccc agt ttc gag ggc    336
Lys Gly Leu Ala Ser Thr Pro Val Thr Arg Arg Pro Ser Phe Glu Gly
            100                 105                 110

aca ggg gaa gca ctc cca tcc tac cac cag ctg ggt ggt gca aac tac    384
Thr Gly Glu Ala Leu Pro Ser Tyr His Gln Leu Gly Gly Ala Asn Tyr
        115                 120                 125

gag ggc ccc gcc gca ctg gag gag atg ccg cgg caa tat tta gac ttt    432
Glu Gly Pro Ala Ala Leu Glu Glu Met Pro Arg Gln Tyr Leu Asp Phe
    130                 135                 140

ctc ttc cct gga gcc gga gcc ggc acc cac ggt gcc cag gct cac cag    480
Leu Phe Pro Gly Ala Gly Ala Gly Thr His Gly Ala Gln Ala His Gln
145                 150                 155                 160

cat cct ccc aaa ggg tac agc aca gca gta gag cca agt gcg cac ttt    528
His Pro Pro Lys Gly Tyr Ser Thr Ala Val Glu Pro Ser Ala His Phe
                165                 170                 175

ccg ggc aca cac tat ggt cgt ggt cat cta cta tcg gag cag tct ggg    576
Pro Gly Thr His Tyr Gly Arg Gly His Leu Leu Ser Glu Gln Ser Gly
            180                 185                 190

tat ggg gtg cag cgc agt tcc tcc ttc cag aac aag acg cca cca gat    624
Tyr Gly Val Gln Arg Ser Ser Ser Phe Gln Asn Lys Thr Pro Pro Asp
        195                 200                 205

gcc tat tcc agc atg gcc aag gcc cag ggt ggc cct ccc gcc agc ctc    672
Ala Tyr Ser Ser Met Ala Lys Ala Gln Gly Gly Pro Pro Ala Ser Leu
    210                 215                 220

acc ttt cct gcc cat gct ggg ctg tac act gcc tcg cac cac aag ccg    720
Thr Phe Pro Ala His Ala Gly Leu Tyr Thr Ala Ser His His Lys Pro
225                 230                 235                 240
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gcg gct acc cca cct ggg gcc cac cca tta cat gtg ttg ggc acc cgg    768
Ala Ala Thr Pro Pro Gly Ala His Pro Leu His Val Leu Gly Thr Arg
                245                 250                 255

ggt ccc acg ttt act ggc gaa agc tct gca cag gct gtg ctg gca ccg    816
Gly Pro Thr Phe Thr Gly Glu Ser Ser Ala Gln Ala Val Leu Ala Pro
            260                 265                 270

tcc agg aac agc ctc aat gct gac ttg tac gag ctg ggc tcc acg gtg    864
Ser Arg Asn Ser Leu Asn Ala Asp Leu Tyr Glu Leu Gly Ser Thr Val
        275                 280                 285

ccc tgg tct gca gct cca ctg gca cgc cgc gac tcg ctg cag aag cag    912
Pro Trp Ser Ala Ala Pro Leu Ala Arg Arg Asp Ser Leu Gln Lys Gln
    290                 295                 300

ggt cta gaa gcc tcg cgg ccg cat gtg gct ttt cgg gct ggc ccc agc    960
Gly Leu Glu Ala Ser Arg Pro His Val Ala Phe Arg Ala Gly Pro Ser
305                 310                 315                 320

agg acc aac tcc ttc aac aac cca caa cct gag ccc tca ctg ccc gcc    1008
Arg Thr Asn Ser Phe Asn Asn Pro Gln Pro Glu Pro Ser Leu Pro Ala
                325                 330                 335

ccc aac acg gtc acc gcc gtg acg gcc gca cac atc ctt cac cct gtg    1056
Pro Asn Thr Val Thr Ala Val Thr Ala Ala His Ile Leu His Pro Val
            340                 345                 350

aag agc gtg cgt gtg ctg cgg ccc gag ccc cag aca gcc gtg ggg ccc    1104
Lys Ser Val Arg Val Leu Arg Pro Glu Pro Gln Thr Ala Val Gly Pro
        355                 360                 365

tcg cac ccc gcc tgg gtg gct gcg ccc aca gca cct gcc act gag agc    1152
Ser His Pro Ala Trp Val Ala Ala Pro Thr Ala Pro Ala Thr Glu Ser
    370                 375                 380

ctg gag acg aag gag ggc agc gca ggc cca cac ccg ctg gat gtg gac    1200
Leu Glu Thr Lys Glu Gly Ser Ala Gly Pro His Pro Leu Asp Val Asp
385                 390                 395                 400

tat ggc ggc tcc gag cgc agg tgc cca ccg cct ccg tat cca aag cac    1248
Tyr Gly Gly Ser Glu Arg Arg Cys Pro Pro Pro Pro Tyr Pro Lys His
                405                 410                 415

ttg ctg ctg ccc agt aag tct gag cag tac agc gtg gac ctg gac agc    1296
Leu Leu Leu Pro Ser Lys Ser Glu Gln Tyr Ser Val Asp Leu Asp Ser
            420                 425                 430

ctg tgc acc agt gtg cag cag agt ctg cga ggg ggc act gat cta gac    1344
Leu Cys Thr Ser Val Gln Gln Ser Leu Arg Gly Gly Thr Asp Leu Asp
        435                 440                 445

ggg agt gac aag agc cac aaa ggt gcg aag gga gac aaa gct ggc aga    1392
Gly Ser Asp Lys Ser His Lys Gly Ala Lys Gly Asp Lys Ala Gly Arg
    450                 455                 460
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gac aaa aag cag att cag acc tcc ccg gtg cct gtc cgc aag aat agc 1440
Asp Lys Lys Gln Ile Gln Thr Ser Pro Val Pro Val Arg Lys Asn Ser
465 470 475 480

aga gat gaa gag aag aga gag tct cgc atc aag agt tac tcc cct tat 1488
Arg Asp Glu Glu Lys Arg Glu Ser Arg Ile Lys Ser Tyr Ser Pro Tyr
485 490 495

gcc ttc aaa ttc ttc atg gag caa cac gtg gag aat gtc atc aaa acc 1536
Ala Phe Lys Phe Phe Met Glu Gln His Val Glu Asn Val Ile Lys Thr
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Tyr Gln Gln Lys Val Ser Arg Arg Leu Gln Leu Glu Gln Glu Met Ala
515 520 525

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Lys Ala Gly Leu Cys Glu Ala Glu Gln Glu Gln Met Arg Lys Ile Leu
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Tyr Gln Lys Glu Ser Asn Tyr Asn Arg Leu Lys Arg Ala Lys Met Asp
545 550 555 560

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Lys Ser Met Phe Val Lys Ile Lys Thr Leu Gly Ile Gly Ala Phe Gly
565 570 575

gaa gtg tgc ctc gct tgt aag ctg gac act cac gct ctg tac gcc atg 1776
Glu Val Cys Leu Ala Cys Lys Leu Asp Thr His Ala Leu Tyr Ala Met
580 585 590

aag act ctc agg aag aag gat gtc ctg aac cgg aat caa gtg gcc cat 1824
Lys Thr Leu Arg Lys Lys Asp Val Leu Asn Arg Asn Gln Val Ala His
595 600 605

gtc aag gct gag agg gac atc ctg gct gaa gca gac aat gag tgg gtg 1872
Val Lys Ala Glu Arg Asp Ile Leu Ala Glu Ala Asp Asn Glu Trp Val
610 615 620

gtc aaa ctc tac tac tcc ttc cag gac aag gac agc ctg tac ttt gtg 1920
Val Lys Leu Tyr Tyr Ser Phe Gln Asp Lys Asp Ser Leu Tyr Phe Val
625 630 635 640

atg gac tac ata cca ggc ggg gat atg atg agc ctg ctg atc agg atg 1968
Met Asp Tyr Ile Pro Gly Gly Asp Met Met Ser Leu Leu Ile Arg Met
645 650 655

gag gtc ttc cct gag cac ctg gcc cgc ttc tac att gca gag ttg acc 2016
Glu Val Phe Pro Glu His Leu Ala Arg Phe Tyr Ile Ala Glu Leu Thr
660 665 670

ctg gcc att gaa agt gtc cac aag atg ggc ttt atc cac cgg gac atc 2064
Leu Ala Ile Glu Ser Val His Lys Met Gly Phe Ile His Arg Asp Ile
675 680 685

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aag cct gac aac ata ctc atc gac ctg gat ggt cat att aag ctg aca     2112
Lys Pro Asp Asn Ile Leu Ile Asp Leu Asp Gly His Ile Lys Leu Thr
    690                 695                 700

gat ttt ggc ctc tgc act gga ttc agg tgg act cac aat tcc aag tac     2160
Asp Phe Gly Leu Cys Thr Gly Phe Arg Trp Thr His Asn Ser Lys Tyr
705                 710                 715                 720

tac cag aaa ggg aac cac atg aga cag gac agc atg gag ccc ggt gac     2208
Tyr Gln Lys Gly Asn His Met Arg Gln Asp Ser Met Glu Pro Gly Asp
                725                 730                 735

ctc tgg gac gat gtt tcc aac tgt cgc tgt gga gac agg tta aag acc     2256
Leu Trp Asp Asp Val Ser Asn Cys Arg Cys Gly Asp Arg Leu Lys Thr
            740                 745                 750

ctg gag cag agg gcg cag aag cag cac cag agg tgc ctg gca cat tct     2304
Leu Glu Gln Arg Ala Gln Lys Gln His Gln Arg Cys Leu Ala His Ser
        755                 760                 765

ctt gtc ggg aca cca aat tac atc gct ccg gag gtg ctt ctc cgc aaa     2352
Leu Val Gly Thr Pro Asn Tyr Ile Ala Pro Glu Val Leu Leu Arg Lys
    770                 775                 780

ggg tac acg cag ctc tgt gac tgg tgg agc gtc ggt gtg att ctc ttt     2400
Gly Tyr Thr Gln Leu Cys Asp Trp Trp Ser Val Gly Val Ile Leu Phe
785                 790                 795                 800

gag atg ctg gtt ggg cag ccg cct ttc ttg gcc ccc acc ccc aca gag     2448
Glu Met Leu Val Gly Gln Pro Pro Phe Leu Ala Pro Thr Pro Thr Glu
                805                 810                 815

acg cag ctg aag gtg atc aac tgg gag agc acg ctg cat atc cct acg     2496
Thr Gln Leu Lys Val Ile Asn Trp Glu Ser Thr Leu His Ile Pro Thr
            820                 825                 830

cag gtg agg ctc agc gct gag gcc cga gac ctc atc acg aag ctg tgc     2544
Gln Val Arg Leu Ser Ala Glu Ala Arg Asp Leu Ile Thr Lys Leu Cys
        835                 840                 845

tgc gcg gct gac tgc cgc ctg ggc agg gat ggg gca gat gac ctc aag     2592
Cys Ala Ala Asp Cys Arg Leu Gly Arg Asp Gly Ala Asp Asp Leu Lys
    850                 855                 860

gca cac ccg ttc ttc aac acc atc gac ttt tcc cgt gac atc cga aag     2640
Ala His Pro Phe Phe Asn Thr Ile Asp Phe Ser Arg Asp Ile Arg Lys
865                 870                 875                 880

cag gct gca ccc tac gtc ccc acc atc agc cac ccc atg gac acc tcc     2688
Gln Ala Ala Pro Tyr Val Pro Thr Ile Ser His Pro Met Asp Thr Ser
                885                 890                 895

aat ttt gac ccg gtg gat gaa gaa agc ccc tgg cac gag gcc agc gga     2736
Asn Phe Asp Pro Val Asp Glu Glu Ser Pro Trp His Glu Ala Ser Gly
            900                 905                 910
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gag agc gcc aag gcc tgg gac acg ctg gcc tcc ccc agc agc aag cat     2784
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        915                 920                 925

cca gag cac gcc ttc tat gag ttc acc ttc cgc agg ttc ttc gat gac     2832
Pro Glu His Ala Phe Tyr Glu Phe Thr Phe Arg Arg Phe Phe Asp Asp
    930                 935                 940

aac ggc tat ccc ttc cgg tgc ccg aag ccc tca gag ccc gca gag agt     2880
Asn Gly Tyr Pro Phe Arg Cys Pro Lys Pro Ser Glu Pro Ala Glu Ser
945                 950                 955                 960

gca gac cca ggg gat gcg gac ttg gaa ggt gcg gcc gag ggc tgc cag     2928
Ala Asp Pro Gly Asp Ala Asp Leu Glu Gly Ala Ala Glu Gly Cys Gln
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ccg gtg tac gtg taagcctcag ttaaccacaa ctcgaggaaa cccaaaatga         2980
Pro Val Tyr Val
            980

gatttctttt cagaagacaa actcaagctt aggaatcctt catttttagt tctggtaaat 3040

gggcaacagg aagagtcaac atgatttcaa attagccctc tgaggacctt cactgcatta 3100

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Ala Ala Glu Val Asn Arg Gln Met Leu Gln Glu Leu Val Asn Ala Ala
        35                  40                  45

Cys Asp Gln Glu Met Ala Gly Arg Ala Leu Thr Gln Thr Gly Ser Arg
    50                  55                  60

Ser Ile Glu Ala Ala Leu Glu Tyr Ile Ser Lys Met Gly Tyr Leu Asp
65                  70                  75                  80

Pro Arg Asn Glu Gln Ile Val Arg Val Ile Lys Gln Thr Ser Pro Gly
                85                  90                  95

Lys Gly Leu Ala Ser Thr Pro Val Thr Arg Arg Pro Ser Phe Glu Gly
            100                 105                 110

Thr Gly Glu Ala Leu Pro Ser Tyr His Gln Leu Gly Gly Ala Asn Tyr
        115                 120                 125
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Glu Gly Pro Ala Ala Leu Glu Glu Met Pro Arg Gln Tyr Leu Asp Phe
130 135 140

Leu Phe Pro Gly Ala Gly Ala Gly Thr His Gly Ala Gln Ala His Gln
145 150 155 160

His Pro Pro Lys Gly Tyr Ser Thr Ala Val Glu Pro Ser Ala His Phe
165 170 175

Pro Gly Thr His Tyr Gly Arg Gly His Leu Leu Ser Glu Gln Ser Gly
180 185 190

Tyr Gly Val Gln Arg Ser Ser Ser Phe Gln Asn Lys Thr Pro Pro Asp
195 200 205

Ala Tyr Ser Ser Met Ala Lys Ala Gln Gly Gly Pro Pro Ala Ser Leu
210 215 220

Thr Phe Pro Ala His Ala Gly Leu Tyr Thr Ala Ser His His Lys Pro
225 230 235 240

Ala Ala Thr Pro Pro Gly Ala His Pro Leu His Val Leu Gly Thr Arg
245 250 255

Gly Pro Thr Phe Thr Gly Glu Ser Ser Ala Gln Ala Val Leu Ala Pro
260 265 270

Ser Arg Asn Ser Leu Asn Ala Asp Leu Tyr Glu Leu Gly Ser Thr Val
275 280 285

Pro Trp Ser Ala Ala Pro Leu Ala Arg Arg Asp Ser Leu Gln Lys Gln
290 295 300

Gly Leu Glu Ala Ser Arg Pro His Val Ala Phe Arg Ala Gly Pro Ser
305 310 315 320

Arg Thr Asn Ser Phe Asn Asn Pro Gln Pro Glu Pro Ser Leu Pro Ala
325 330 335

Pro Asn Thr Val Thr Ala Val Thr Ala Ala His Ile Leu His Pro Val
340 345 350

Lys Ser Val Arg Val Leu Arg Pro Glu Pro Gln Thr Ala Val Gly Pro
355 360 365

Ser His Pro Ala Trp Val Ala Ala Pro Thr Ala Pro Ala Thr Glu Ser
370 375 380

Leu Glu Thr Lys Glu Gly Ser Ala Gly Pro His Pro Leu Asp Val Asp
385 390 395 400

Tyr Gly Gly Ser Glu Arg Arg Cys Pro Pro Pro Pro Tyr Pro Lys His
405 410 415

Leu Leu Leu Pro Ser Lys Ser Glu Gln Tyr Ser Val Asp Leu Asp Ser
420 425 430

Leu Cys Thr Ser Val Gln Gln Ser Leu Arg Gly Gly Thr Asp Leu Asp
435 440 445

Gly Ser Asp Lys Ser His Lys Gly Ala Lys Gly Asp Lys Ala Gly Arg
450 455 460

Asp Lys Lys Gln Ile Gln Thr Ser Pro Val Pro Val Arg Lys Asn Ser
465 470 475 480

Arg Asp Glu Glu Lys Arg Glu Ser Arg Ile Lys Ser Tyr Ser Pro Tyr
485 490 495

Ala Phe Lys Phe Phe Met Glu Gln His Val Glu Asn Val Ile Lys Thr
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Tyr Gln Gln Lys Val Ser Arg Arg Leu Gln Leu Glu Gln Glu Met Ala
515 520 525

Lys Ala Gly Leu Cys Glu Ala Glu Gln Glu Gln Met Arg Lys Ile Leu
530 535 540

Tyr Gln Lys Glu Ser Asn Tyr Asn Arg Leu Lys Arg Ala Lys Met Asp
545 550 555 560

Lys Ser Met Phe Val Lys Ile Lys Thr Leu Gly Ile Gly Ala Phe Gly
565 570 575

Glu Val Cys Leu Ala Cys Lys Leu Asp Thr His Ala Leu Tyr Ala Met
580 585 590

Lys Thr Leu Arg Lys Lys Asp Val Leu Asn Arg Asn Gln Val Ala His
595 600 605

Val Lys Ala Glu Arg Asp Ile Leu Ala Glu Ala Asp Asn Glu Trp Val
610 615 620

Val Lys Leu Tyr Tyr Ser Phe Gln Asp Lys Asp Ser Leu Tyr Phe Val
625 630 635 640

Met Asp Tyr Ile Pro Gly Gly Asp Met Met Ser Leu Leu Ile Arg Met
645 650 655

Glu Val Phe Pro Glu His Leu Ala Arg Phe Tyr Ile Ala Glu Leu Thr
660 665 670

Leu Ala Ile Glu Ser Val His Lys Met Gly Phe Ile His Arg Asp Ile
675 680 685

Lys Pro Asp Asn Ile Leu Ile Asp Leu Asp Gly His Ile Lys Leu Thr
690 695 700

Asp Phe Gly Leu Cys Thr Gly Phe Arg Trp Thr His Asn Ser Lys Tyr
705 710 715 720

Tyr Gln Lys Gly Asn His Met Arg Gln Asp Ser Met Glu Pro Gly Asp
725 730 735

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Leu Trp Asp Asp Val Ser Asn Cys Arg Cys Gly Asp Arg Leu Lys Thr
            740                 745                 750

Leu Glu Gln Arg Ala Gln Lys Gln His Gln Arg Cys Leu Ala His Ser
        755                 760                 765

Leu Val Gly Thr Pro Asn Tyr Ile Ala Pro Glu Val Leu Leu Arg Lys
    770                 775                 780

Gly Tyr Thr Gln Leu Cys Asp Trp Trp Ser Val Gly Val Ile Leu Phe
785                 790                 795                 800

Glu Met Leu Val Gly Gln Pro Pro Phe Leu Ala Pro Thr Pro Thr Glu
                805                 810                 815

Thr Gln Leu Lys Val Ile Asn Trp Glu Ser Thr Leu His Ile Pro Thr
            820                 825                 830

Gln Val Arg Leu Ser Ala Glu Ala Arg Asp Leu Ile Thr Lys Leu Cys
        835                 840                 845

Cys Ala Ala Asp Cys Arg Leu Gly Arg Asp Gly Ala Asp Asp Leu Lys
    850                 855                 860

Ala His Pro Phe Phe Asn Thr Ile Asp Phe Ser Arg Asp Ile Arg Lys
865                 870                 875                 880

Gln Ala Ala Pro Tyr Val Pro Thr Ile Ser His Pro Met Asp Thr Ser
                885                 890                 895

Asn Phe Asp Pro Val Asp Glu Glu Ser Pro Trp His Glu Ala Ser Gly
            900                 905                 910

Glu Ser Ala Lys Ala Trp Asp Thr Leu Ala Ser Pro Ser Ser Lys His
        915                 920                 925

Pro Glu His Ala Phe Tyr Glu Phe Thr Phe Arg Arg Phe Phe Asp Asp
    930                 935                 940

Asn Gly Tyr Pro Phe Arg Cys Pro Lys Pro Ser Glu Pro Ala Glu Ser
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gagtgaatca agtgaaaata cgtcggcaaa tatcagcgaa ttgtcgtcaa aaggcaagga 180

aaaacggaga aaaagaggaa aagcaataag tgccgtgtgt gggaaacgcg aaaaaggcga 240

gaacaaagag gcgaaaagcg aggaaattgc gtggaaaacg tggaaaacgc gaagaagcga 300

agctccaagt tggccgccat cgattcgtgc gtaggatcaa ttaagattcc gagtggtcga 360

gaatcggctc aaatcaaatt aaaatcaact aatattttgg tattcagata ttcaaatgga 420

attcattcat cgcctgcgac ttttattcgg atctgccaac tatttttgaa tttgaattgt 480

gtgtctgcgg ctggcgcaga atctctgata aagcagagga ataaaatcgg aagaacaaca 540

aatacaaata caaatgaaat gcggggagca gtatttacat gccaaatgaa tgctggatag 600

gcgaaagggg gggtttctct tataatgcaa atgtgaatgt gaatgcgaat gcgaatgcga 660

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gagataataa agcatcctaa aacccgcgcc ttagttcgtt ttagtctcgc cacggatata 840

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caaatcaaat tcccggccgc cgatgtgccc cagtgtgtgt gcgtgtgtgt gtgtgtgtgc 1020

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cacagggcga agcagcagca tc atg cat cca gcg ggc gaa aaa agg ggc ggt 1132
Met His Pro Ala Gly Glu Lys Arg Gly Gly
1 5 10

cgc ccc aat gat aaa tac acg gcg gaa gcc ctc gag agc atc aag cag 1180
Arg Pro Asn Asp Lys Tyr Thr Ala Glu Ala Leu Glu Ser Ile Lys Gln
15 20 25

gac cta acc cga ttt gaa gta caa aat aac cat agg aat aat cag aat 1228
Asp Leu Thr Arg Phe Glu Val Gln Asn Asn His Arg Asn Asn Gln Asn
30 35 40

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Tyr Thr Pro Leu Arg Tyr Thr Ala Thr Asn Gly Arg Asn Asp Ala Leu
45 50 55

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Thr Pro Asp Tyr His His Ala Lys Gln Pro Met Glu Pro Pro Pro Ser
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Ala Ser Pro Ala Pro Asp Val Val Ile Pro Pro Pro Pro Ala Ile Val
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Gly Gln Pro Gly Ala Gly Ser Ile Ser Val Ser Gly Val Gly Val Gly
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Val Val Gly Val Ala Asn Gly Arg Val Pro Lys Met Met Thr Ala Leu
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atg cca aac aaa ctg atc cgg aag ccg agc atc gaa cgg gac acg gcg     1516
Met Pro Asn Lys Leu Ile Arg Lys Pro Ser Ile Glu Arg Asp Thr Ala
        125                 130                 135

agc agt cac tac ctg cgc tgc agt ccg gct ctg gac tcc gga gcc ggt     1564
Ser Ser His Tyr Leu Arg Cys Ser Pro Ala Leu Asp Ser Gly Ala Gly
    140                 145                 150

agc tcc cga tcg gac agc ccc cat tcg cac cac acc cac cag ccg agc     1612
Ser Ser Arg Ser Asp Ser Pro His Ser His His Thr His Gln Pro Ser
155                 160                 165                 170

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Ser Arg Thr Val Gly Asn Pro Gly Gly Asn Gly Gly Phe Ser Pro Ser
                175                 180                 185

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Pro Ser Gly Phe Ser Glu Val Ala Pro Pro Ala Pro Pro Pro Arg Asn
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Pro Thr Ala Ser Ser Ala Ala Thr Pro Pro Pro Pro Val Pro Pro Thr
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Ser Gln Ala Tyr Val Lys Arg Arg Ser Pro Ala Leu Asn Asn Arg Pro
    220                 225                 230

ccg gcg ata gcg cca ccc act cag cga ggc aac tca cct gta ata acc     1852
Pro Ala Ile Ala Pro Pro Thr Gln Arg Gly Asn Ser Pro Val Ile Thr
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Gln Asn Gly Leu Lys Asn Pro Gln Gln Gln Leu Thr Gln Gln Leu Lys
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tcc ctg aac cta tac cca ggc gga ggc agt gga gca gtg gtg gag cca     1948
Ser Leu Asn Leu Tyr Pro Gly Gly Gly Ser Gly Ala Val Val Glu Pro
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ccg ccg ccc tac cta att caa ggc gga gcc gga gga gca gca ccg ccg     1996
Pro Pro Pro Tyr Leu Ile Gln Gly Gly Ala Gly Gly Ala Ala Pro Pro
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315 320 325 330

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Tyr Ser Ala Thr Ser Ala Gly Ser Pro Ser Pro Ile Thr Val Ser Leu
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Pro Pro Ala Pro Leu Ala Lys Pro Gln Pro Arg Val Tyr Gln Ala Arg
350 355 360

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Ser Gln Gln Pro Ile Ile Met Gln Ser Val Lys Ser Thr Gln Val Gln
365 370 375

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Lys Pro Val Leu Gln Thr Ala Val Ala Arg Gln Ser Pro Ser Ser Ala
380 385 390

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415 420 425

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Pro Thr Thr Pro Pro Leu Val Gly Leu Asn Ser Lys Pro Asn Cys Leu
445 450 455

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Glu Pro Pro Ser Tyr Ala Lys Ser Met Gln Ala Lys Ala Ala Thr Val
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575 580 585

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Pro Ile Pro Glu Arg Lys Lys Ile Ser Lys Glu Lys Glu Glu Glu Arg
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aag gag ttc cgc atc agg cag tac tcg ccg caa gcc ttc aag ttc ttc 3052
Lys Glu Phe Arg Ile Arg Gln Tyr Ser Pro Gln Ala Phe Lys Phe Phe
635 640 645 650

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655 660 665

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Tyr Arg Lys Asn Gln Leu Glu Lys Glu Met His Lys Val Gly Leu Pro
670 675 680

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Asp Gln Thr Gln Ile Glu Met Arg Lys Met Leu Asn Gln Lys Glu Ser
685 690 695

aac tac att cga ttg aag cgc gcc aag atg gac aag agc atg ttc gtc 3244
Asn Tyr Ile Arg Leu Lys Arg Ala Lys Met Asp Lys Ser Met Phe Val
700 705 710

aaa ctg aag ccc att gga gtg ggt gca ttt ggc gag gta acg ctg gtg 3292
Lys Leu Lys Pro Ile Gly Val Gly Ala Phe Gly Glu Val Thr Leu Val
715 720 725 730

agc aaa atc gat acc tcg aac cat ttg tat gcg atg aaa acc ctg cgg 3340
Ser Lys Ile Asp Thr Ser Asn His Leu Tyr Ala Met Lys Thr Leu Arg
735 740 745

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            750                 755                 760

agg gat atc ctc gcg gaa gcc gac aat aac tgg gtg gtg aag ttg tac    3436
Arg Asp Ile Leu Ala Glu Ala Asp Asn Asn Trp Val Val Lys Leu Tyr
        765                 770                 775

tac agc ttc cag gac aag gat aat ctg tac ttt gtg atg gac tac ata    3484
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19068

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :Please See Extra Sheet.
US CL :424/130.1; 536/23.5; 514/1, 2, 44; 800/3, 13, 14, 21, 25
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/130.1; 536/23.5; 514/1, 2, 44; 800/3, 13, 14, 21, 25

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST, STN, MEDLINE, CAPLUS, BIOSIS, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 96/30402 A1 (YALE UNIVERSITY) 03 October 1996, entilre document, especially pages 158-163.	1-5 6-113
Y	WO 95/31722 A1 (LIGAND PHARMACEUTICALS, INC.) 23 November 1995, entire document, especially page 50.	6-37
Y, P	TAO et al. Human homologue of the Drosophila melanogaster lats tumor suppressor modulates CDC2 activity, Nature Genetics, February 1999, Vol 21, No. 2, page 177-181, entire document.	68-113

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 OCTOBER 1999	16 DEC 1999

Name and mailing address of the ISA/US
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Authorized officer
SHIN-LIN CHEN
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	ST. JOHN et al. Mice Deficient of Lats1 Develop Soft-Tissue Sarcomas, Ovarian Tumors and Pituitary Dysfunction, Nature Genetics, February 1999, Vol 21, No. 2, page 182-186, entire document.	1-37

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19068

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19068

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 39/395; C07H 21/04; A01N 61/00, 37/18, 43/04; C12N 5/00, 15/00

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classificati n 6 : G01N 33/50, 33/68

A1

(11) International Publicati n Number: WO 95/31722

(43) International Publicati n Date: 23 November 1995 (23.11.95)

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(30) Priority Data:
08/245,470 18 May 1994 (18.05.94) US

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(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published

With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: SCREENING FOR CYTOKINE MODULATORS

(57) Abstract

This invention provides a method for screening for agents useful for treatment of diseases and pathological conditions affected by cytokines. These agents interact directly or indirectly with an intracellular receptor, which in turn modulates the binding of a rel-like protein, a rel-like protein complex, or other transcriptional proteins to a rel site on the promoter of a cytokine gene. The intracellular receptor can be the estrogen receptor, retinoid acid receptors, retinoid X receptors, glucocorticoid receptor, progesterone receptors, androgen receptor, thyroid hormone receptors, or vitamin D receptor. The select agents can be used to treat osteoporosis, rheumatoid arthritis, inflammation, psoriasis, Kaposi's sarcoma, septic shock and multiple myeloma.

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GA	Gabon				

SCREENING FOR CYTOKINE MODULATORS

FIELD OF THE INVENTION

This invention relates to a method for screening for agents useful for treatment of diseases and pathological conditions affected by cytokines and novel agents identified using such screening method.

BACKGROUND OF THE INVENTION

Cytokines are a group of molecules capable of signalling cellular development. Aberrant expression of cytokines is known to be associated with pathological conditions including autoimmune diseases, septic shock, rheumatoid arthritis, psoriasis, inflammation, postmenopausal osteoporosis, and some cancers. Common treatment for these pathological conditions are retinoids, immunosuppressants, glucocorticoids and other steroid drugs. Estrogens are specifically employed in the prevention of postmenopausal osteoporosis.

Steroids and related hormone drugs exert their therapeutic effects by binding to a superfamily of intracellular receptors (IRs), which are regulators of gene transcription. IRs can function as activators as well as repressors of specific cytokine genes. The activity of IRs is controlled by hormones or other ligands that bind to the IRs.

The classical mechanism of transcriptional regulation by IRs involves binding of the IRs to specific response elements in the promoters of the regulated genes, for example, the binding of the estrogen receptor to its response site in the vitellogenin gene (Klein-Hitpass et al., Cell 46:1053-1061, 1986). More recently a different mechanism of IRs function has been described in glucocorticoid receptor mediated AP-1 transcription regulation that does not

require direct DNA-binding of the IRs (Yang-Yen et al., Cell 62:1205-1215, 1990).

Although steroid drugs have been shown to repress the level of certain cytokines, a lack of tissue specificity and side effects of the steroids may limit their use as therapeutic agents. These side effects may be reduced or completely avoided with more specifically acting compounds.

Pfahl and Karin (PCT publication, WO 92/07072, 1992) describes a method of screening a sample for ligands which bind to a nuclear receptor to form a complex which binds or interferes with a non rel-like protein AP-1 or an AP-1 component.

SUMMARY OF THE INVENTION

The present invention relates to a method for identifying new therapeutic agents and for using these agents to treat diseases and conditions affected by cytokines, such as, but not limited to, osteoporosis, rheumatoid arthritis, inflammation, psoriasis, septic shock, Kaposi's sarcoma and multiple myeloma. This method makes it possible to screen large collections of natural, semisynthetic, or synthetic compounds for therapeutic agents that affect the transcription of a cytokine through an intracellular receptor mediated pathway.

By "cytokine" is generally meant a secreted protein which acts as a chemical mediator of cellular regulation.. More specifically, it is meant a diverse groups of soluble polypeptides such as growth factors and hormones that control the growth, differentiation and function of cells, including, but not limited to, GM-CSF, G-CSF, IL-2, IL-6, IL-8, and IL-11.

The present invention relates to the determination that inhibition of interleukin 6 (IL-6) expression by estrogen-estrogen receptor complex is mediated through

the control of the transcriptional activity of NFκB or closely related proteins on the IL-6 promoter. This mechanism does not involve direct binding of ER to IL-6 promoter but controls the DNA-binding properties of the activated NFκB and possible other members of the rel-family of proteins to their specific response elements (i.e., rel site) on the IL-6 promoter.

Because NFκB is involved in the regulation of genes encoding various cytokines and their receptors, viral proteins, and proteins involved in the acute-phase response, the regulation of NFκB activity by estrogen and possible other hormones is of general importance (see generally Baeuerle, Biochemica et Biophysica Acta, 1072:63-80, 1993, incorporated by reference herein). For example, retinoic acid treatment, which strongly inhibits IL-6 expression in +/+LDA11 cells and other tissues (Gross, V., P. M. Villiger, B. Zhang, and M. Lotz, 1993, "Retinoic acid inhibits interleukin-1-induced cytokine synthesis in human monocytes," J. Leukoc. Biol. 54:125-132), has the same effect as estrogen on the NFκB related complexes with the IL-6 promoter. This suggests a general pathway of transcriptional regulation involving cross-talk between members of the intracellular receptor family and the NFκB transcription factors.

The above determination allows for the screening of drugs that specifically influence genes controlled by the rel-transcription factors, i.e. genes involved in inflammation, sepsis, skin and kidney disorders, osteoporosis, certain cancers, and hematopoietic dysfunctions without the side effects of known steroid drugs. The diseases listed are usually correlated with aberrant expression of cytokines such as IL-1, TNFα, IL-6, IL-8 that are under the control of NFκB or other rel proteins.

Thus, the present invention features a method for identifying agents which, by activating an intracellular receptor, cause a significant reduction in the binding of a rel-like protein or other transcriptional protein to the rel site on the promoter of a cytokine gene or a portion of the promoter, thereby reducing the transcription of the cytokine.

By "intracellular receptor" is meant an intracellular transcription factor whose activity is regulated by binding of small molecules, including, but not limited to, estrogen receptor, retinoid acid receptors, retinoid X receptors, glucocorticoid receptor, progesterone receptors, androgen receptor, thyroid hormone receptors, and vitamin D receptor.

By "rel-like protein" is meant a protein or a protein complex of the rel family that share a homology in the rel domain and is involved in gene regulation (see Liou and Baltimore, Current Opinion in Cell Biology, 5:477-487, 1993, incorporated by reference herein), including, but not limited to, NFκB, Lyt-10, c-rel, and relB.

By "transcriptional protein" is meant a cytoplasmic or nuclear protein that, when activated, bind a promoter either directly, or indirectly through a complex of proteins to modulate the transcription activity of the promoter.

By "rel site" is meant a DNA sequence that serves as a binding site for rel-like proteins or complexes comprising one or more rel-like proteins, including, but not limited to, κB motifs identified in Baeuerle, Biochemica et Biophysica Acta, 1072:63-80, 1993, incorporated by reference herein, such as the NFκB binding site on IL-6 promoter.

By "promoter" is meant a DNA regulatory region capable of binding directly or indirectly to RNA polymerase in a cell and initiating transcription of a

downstream (3′ direction) coding sequence. A promoter of a DNA construct, including an oligonucleotide sequence according to the present invention may be linked to a heterologous gene when the presence of the promoter influences transcription from the heterologous gene, including genes for reporter sequences such as luciferase, chloramphenicol acetyl transferase, β-galactosidase and secreted placental alkaline phosphatase.

In a preferred embodiment, the assay is conducted in a whole cell system that has an intracellular receptor which is the target of the screened agent, a promoter or a portion of a promoter with a rel site and a rel-like protein or other transcription protein that binds to the rel site; wherein the intracellular receptor modulates the binding of the rel-like protein or the transcription protein to the rel site. The intracellular receptor, the promoter or a portion of the promoter, or the protein that binds to the rel site may either be endogenous to the cell or transfected into the cell.

In another preferred embodiment, the assay is conducted in an extract of cell having an intracellular receptor, a promoter or a portion of a promoter, with a rel site and a rel-like protein or other protein that binds to the rel site; wherein the intracellular receptor modulates the binding of the rel-like protein or the transcription protein to the rel site.

The binding of the rel-like protein or other transcription protein to the rel site may be measured by techniques known to those skilled in the art, including, but not limited to, mobility shift assay, co-transfection assay, and expression of a reporter gene linked to the promoter.

In a further preferred embodiment, the promoter is activated by an effector, including, but not limited to, tumor necrosis factor, interleukin-1, viruses,

endotoxins, phorbol esters, epidermal growth factor, leukemia inhibitor factor and cAMP agonists.

By "effector" is meant an agent that stimulates the expression of a cytokine to a measurable level. An effector may be endogenously produced in a cell or exogenously added to a cell

In another further preferred embodiment, the claimed assay is conducted in a system including an estrogen receptor, an interleukin 6 promoter or a portion of an IL-6 promoter and NFκB; wherein ER modulates the binding of NFκB or related proteins to the NFκB site on the IL-6 promoter.

The agents discovered by the above assay may either interact directly with an intracellular receptor, or modulate the interaction of a ligand with the intracellular receptor. Thus, in an even further preferred embodiment, a ligand for the intracellular receptor is included in the assay.

While steroids and steroid analogs may exemplify agents identified by the present invention, Applicant is particularly interested in the identification of agents of low molecular weight (less than 10,000 daltons, preferably less than 5,000, and most preferably less than 1,000) which can be readily formulated as useful therapeutic agents.

Such agents can then be screened to ensure that they are specific to tissues with cytokine inflicted pathological conditions with little or no effect on healthy tissues such that the agents can be used in a therapeutic or prophylactic manner. If such agents have some effect on healthy tissues they may still be useful in therapeutic treatment, particularly in those diseases which are life threatening, such as Kaposi's sarcoma or multiple myeloma.

Once isolated, a candidate agent can be put in pharmaceutically acceptable formulations, and used for

specific treatment of diseases and pathological conditions with little or no effect on healthy tissues.

Other features and advantages of the invention will be apparent from the following detailed description of the invention, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows IL-1 and TNFα induced complex formation on the proximal IL-6 promoter.

Figure 2 shows that several distinct NFκB-related complexes induced by IL-1 and TNFα are modulated by estrogen.

Figure 3 shows the effects of estrogen agonist and antagonist, and inhibitors of protein synthesis and protein kinase C on the formation of NFκB-related complexes.

Figure 4 shows the binding characteristics of proteins in NFκB-related complexes with NFκB oligonucleotides.

Figure 5 shows NFκB related proteins in complexes A, B, and C.

DETAILED DESCRIPTION OF THE INVENTION

A number of cytokines, including IL-6, Il-8 and IL-11, have related biological effects, i.e., effects on cellular defense in response to infection by stimulating the immune and the acute-phase response and on bone metabolism by increasing bone resorption. Aberrant expression of any of these cytokines results in similar pathological conditions, e.g., all cytokines listed are involved in septic shock. In another example, excessive production IL-8, like IL-6, may be involved in the pathogenesis of several types of inflammatory reactions, particularly neutrophil-dependent tissue damages. These cytokines have similar promoter structures, e.g., their promoters contain binding sites for NFκB or other rel

proteins. It is therefore likely that not only IL-6 but also the other cytokines mentioned above can be targeted by drugs that modulate the binding of NFκB or other rel proteins to their promoter sites through the intracellular receptors.

Interleukin 6 and Diseases

Interleukin-6 (IL-6) is a pleiotropic cytokine that is secreted by many different cells, including monocytes, macrophages, certain B-lymphocytes and T-lymphocytes, glial cells, fibroblasts, osteoblasts, and stromal cells (reviewed in references Hirano, T., (1992) "The biology of interleukin-6," Chem. Immunol. 51:153-180.; Kishimoto, T. (1989) "The biology of interleukin-6," Blood 74:1-10.; Kishimoto, T., M. Hibi, M. Murakami, M. Narazaki, M. Saito, and T. Taga (1992) "The molecular biology of interleukin 6 and its receptor," Ciba Found. Symp. 167:5-16; discussion 16-23; and Wolvekamp, M. C., and R. L. Marquet (1990) "Interleukin-6: historical background, genetics and biological significance," Immunol. Lett. 24:1-9). Due to its induction in response to tissue injury, inflammation and infection, IL-6 function is mainly associated with the host's immune and acute phase responses.

IL-6 is an important mediator of intercellular communication not only under pathological conditions but also under normal physiological conditions. It is involved in neural differentiation (Satoh, T., S. Nakamura, T. Taga, T. Matsuda, T. Hirano, T. Kishimoto, and Y. Kaziro (1988) "Induction of neuronal differentiation in PC12 cells by B-cell stimulatory factor 2/interleukin 6," Mol. Cell Biol. 8:3546-3549), and proliferation and differentiation during hematopoiesis (Ikebuchi, K., G.G. Wong, S.C. Clark, J.N. Ihle, Y. Hirai, and M. Ogawa (1987) "Interleukin 6 enhancement of interleukin 3-dependent proliferation of

multipotential hemopoietic progenitors," Proc. Natl. Acad. Sci. U.S.A. 84:9035-9039). However, elevated IL-6 expression is usually associated with disease (Yu, X.P., T. Bellido, N. Rice, and S.C. Manolagas (1993).

IL-6 expression is tightly controlled by other factors. Depending on the particular cell type, it can be activated by various stimuli, including tumor necrosis factor (TNFα) and interleukin-1 (IL-1), viruses, endotoxin (lipopolysaccharides), phorbol esters, epidermal growth factor (EGF), leukemia inhibitor factor (LIF), and cAMP agonists.

These effectors exhibit their activity through transcriptional effects on the IL-6 promoter as shown by transfection studies (Gruss, H.J., M.A. Brach, and F. Herrmann (1992) "Involvement of nuclear factor-kappa B in induction of the interleukin-6 gene by leukemia inhibitory factor," Blood 80:2563-2570; Ray, A., S.B. Tatter, L.T. May, and P.B. Sehgal (1988) "Activation of the human "beta 2-interferon/hepatocyte-stimulating factor/interleukin 6" promoter by cytokines, viruses, and second messenger agonists," Proc. Natl. Acad. Sci. U.S.A. 85:6701-6705). By sequence comparison several potential transcriptional control elements have been identified in the IL-6 promoter, including a cAMP response element, an AP-1 binding site, and binding elements for the transcription factors NF-IL6 (C/EBPB, LAP, AGP/EBP) and NFκB (Isshiki, H., S. Akira, O. Tanabe, T. Nakajima, T. Shimamoto, T. Hirano, and T. Kishimoto (1990) "Constitutive and interleukin-1 (IL-1)-inducible factors interact with the IL-1-responsive element in the IL-6 gene," Mol. Cell Biol. 10:2757-2764).

Direct binding of NF-IL6 and NFκB to the IL-6 promoter has been established (Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto (1990) "A

nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family," EMBO J. 9:1897-1906; Libermann, T.A. and D. Baltimore, (1990) "Activation of interleukin-6 gene expression through the NF-kappa B transcription factor," Mol. Cell Biol. 10:2327-2334). NF-IL6 belongs to the C/EBP family of leucine zipper proteins. It is induced by IL-1, IL-6 and lipopolysaccharide (LPS), and has been shown to interact with its binding site on the IL-6 promoter and to activate IL-6 expression (Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto, (1990) "A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family," EMBO J. 9:1897-1906; Chang, C.J., T.T. Chen, H.Y. Lei, D.S. Chen, and S.C. Lee (1990), "Molecular cloning of a transcription factor, AGP/EBP, that belongs to members of the C/EBP family," Mol. Cell Biol. 10:6642-6653; Descombes, P., M. Chojkier, S. Lichtsteiner, E. Falvey, and U. Schibler (1990) "LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein," Genes Dev. 4:1541-1551; Descombes, P., M. Chojkier, S. Lichtsteiner, E. Falvey, and U. Schibler (1990) "LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein," Genes Dev. 4:1541-1551; Poli, V., F.P. Mancini, and R. Cortese (1990) "IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP," Cell 63:643-653). NFκB is a transcription factor that was originally identified as a heterodimeric complex consisting of a 50 kD protein (p50) and a 65 kd protein (p65) that binds an element in the immunoglobulin kappa light chain enhancer. Both proteins reveal a high

homology to the Drosophila morphogen *dorsal* and to the c-rel proto-oncogeny product. The p65 subunit is also functionally related to c-rel (reviewed in references Baeuerle, P. A. (1991) "The inducible transcription activator NF-kappa B: regulation by distinct protein subunits" Biochim. Biophys. Acta 1072:63-80; Blank, V., P. Kourilsky, and A. Israel (1992) "NF-kappa B and related proteins: Rel/dorsal homologies meet ankyrin-like repeats," Trends. Biochem. Sci. 17:135-140; and Liou, H.C. and D. Baltimore (1993) "Regulation of the NF-kappa B/rel transcription factor and I kappa B inhibitor system," Curr. Opin. Cell Biol. 5:477-487). Recently, additional proteins (p49/p52 and relB/p68) have been identified that are functionally related to p50 and p65 (Henkel, T., T. Machleidt, I. Alkalay, M. Kronke, Y. Ben-Neriah, and P.A. Baeuerle (1993) "Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B," Nature 365:182-185; Perkins, N.D., R.M. Schmid, C.S. Duckett, K. Leung, N.R. Rice, and G.J. Nabel (1992) "Distinct combinations of NF-kappa B subunits determine the specificity of transcriptional activation," Proc. Natl. Acad. Sci. U.S.A. 89:1529-1533; Ryseck, R.P., P. Bull, M. Takamiya, V. Bours, U. Siebenlist, P. Dobrzanski, and R. Bravo (1992) "RelB, a new Rel family transcription activator that can interact with p50-NF-kappa B," Mol. Cell Biol. 12:674-684; Ryseck, R.P., P. Bull, M. Takamiya, V. Bours, U. Siebenlist, P. Dobrzanski, and R. Bravo (1992) "RelB, a new Rel family transcription activator that can interact with p50-NF-kappa B," Mol. Cell Biol. 12:674-684; Schmid, R.M., N.D. Perkins, C.S. Duckett, P.C. Andrews, and G.J. Nabel (1991) "Cloning of an NF-kappa B subunit which stimulates HIV transcription in synergy with p65," Nature 352:733-736). NFκB is located in the cytosol complexes with an inhibitory protein of the IκB family. Upon induction, NFκB

dissociates from IκB and translocates into the nucleus where it binds and activates specific promoters (Baeuerle, P.A. and D. Baltimore (1988) "I kappa B: a specific inhibitor of the NF-kappa B transcription factor," Science 242:540-546; Ghosh, S. and D. Baltimore (1990) "Activation in vitro of NF-kappa B by phosphorylation of its inhibitor I kappa B," Nature 344:678-682). Binding of NFκB-like factors to the consensus site of the IL-6 promoter is induced by IL-1, TNFα, LIF, LPS and phorbol esters, varying with the particular cell type (Gruss, H.J., M.A. Brach, and F. Herrmann (1992) "Involvement of nuclear factor-kappa B in induction of the interleukin-6 gene by leukemia inhibitory factor," Blood 80:2563-2570; Libermann, T.A. and D. Baltimore (1990) "Activation of interleukin-6 gene expression through the NF-kappa B transcription factor," Mol. Cell Biol. 10:2327-2334; Shimizu, H., K. Mitomo, T. Watanabe, S. Okamoto, and K. Yamamoto (1990) "Involvement of a NF-kappa B-like transcription factor in the activation of the interleukin-6 gene by inflammatory lymphokines," Mol. Cell Biol. 10:561-568; Zhang, Y.H., J.X. Lin, and J. Vilcek (1990) "Interleukin-6 induction by tumor necrosis factor and interleukin-1 in human fibroblasts involves activation of a nuclear factor binding to a kappa B-like sequence," Mol. Cell Biol. 10:3818-3823).

Unregulated expression of IL-6 is linked to a number of diseases (Bauer, J. and F. Herrmann (1991) "Interleukin-6 in clinical medicine," Ann. Hematol. 62:203-210; Hirano, T. (1992) "Interleukin-6 and its relation to inflammation and disease," Clin. Immunol. Immunopathol. 62:S60-S65) such as postmenopausal osteoporosis after loss of ovarian function (Roodman, G.D. (1992) "Interleukin-6: an osteotropic factor?" J. Bone Miner. Res. 7:475-478). Ex vivo cultures of bone marrow from ovariectomized mice show an increase of

osteoclastogenesis compared with cultures from sham-operated animals. This increase in osteoclast development can be prevented by injection of an anti-IL-6 antibody or by administration of estrogen (Jilka, R.L., G. Hangoc, G. Girasole, G. Passeri, D.C. Williams, J.S. Abrams, B. Boyce, H. Broxmeyer, and S.C. Manolagas (1992) "Increased osteoclast development after estrogen loss: mediation by interleukin-6," Science 257:88-91). In mice that carry a null mutation for IL-6, ovariectomy does not affect bone volume or osteoclast number as seen with normal mice (Balena, R., F. Costantini, M. Yamamoto, A. Markatos, R. Cortese, G.A. Rodan, and V. Poli (1993) "Mice with IL-6 gene knock-out do not lose cancellous bone after ovariectomy," J. Bone Miner. Res. 8:S130 [Abstract]).

Regulation of Interleukin 6 by Estrogen

Estrogen has been found to inhibit IL-6 expression in bone-derived stromal cell lines and osteoblastic cells from rats and mice as well as in nontransformed human bone cells (Girasole, G., R.L. Jilka, G. Passeri, S. Boswell, G. Boder, D.C. Williams, and S.C. Manolagas (1992) "17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens," J. Clin. Invest. 89:883-891). This effect of estrogen on IL-6 expression is not restricted to bone tissue but has also been shown for uterine cells (Jacobs, A.L., P.B. Sehgal, J. Julian, and D.D. Carson (1992) "Secretion and hormonal regulation of interleukin-6 production by mouse uterine stromal and polarized epithelial cells cultured in vitro," Endocrinology 131:1037-1046; Tabibzadeh, S.S., U. Santhanam, P.B. Sehgal, and L.T. May (1989) "Cytokine-induced production of IFN-beta 2/IL-6 by freshly explanted human endometrial stromal cells. Modulation by estradiol-17 beta," J. Immunol. 142:3134-

3139). There are only a few other genes known to be negatively regulated by estrogen agonists (Adler, S., M.L. Waterman, X. He, and M.G. Rosenfeld (1988) "Steroid receptor-mediated inhibition of rat prolactin gene expression does not require the receptor DNA-binding domain," Cell 52:685-695; Ree, A.H., B.F. Landmark, W. Eskild, F.O. Levy, H. Lahooti, T. Jahnsen, A. Aakvaag, and V. Hansson (1989) "Autologous down-regulation of messenger ribonucleic acid and protein levels for estrogen receptors in MCF-7 cells: an inverse correlation to progesterone receptor levels," Endocrinology 124:2577-2583).

To investigate the mechanism of the estrogen effect, Applicant performed a series of DNA-binding experiments using the human IL-6 promoter. Co-transfection studies showed that the proximal 225 bps of the IL-6 promoter mediate both the induction of the reporter gene by IL-1 and TNFα as well as the repression by estradiol. The repression by estradiol also required the expression of the estrogen receptor (ER).

Using gel retardation assays, no specific binding of the ER to the proximal 225 bp could be detected. However, nuclear extracts from +/+LDA11 bone marrow stromal cells that revealed IL-6 regulation by IL-1, TNFα, and estradiol showed an induced complex with a -225 to -52 promoter fragment when the cell were treated with IL-1 and TNFα. Induction of the complex was fast (10 minutes) but transient. Pretreatment of the cells with estradiol increased the intensity as well as the mobility of the complex.

To identify the proteins involved in the formation of the complex, antibody supershift experiments were carried out using antibodies against factors with potential binding sites in this promoter fragment including c-jun, NF-IL6, c-rel, and NFκB p50 and p65

proteins. Only anti-p50 and anti-p65 had an effect and abolished the formation of the induced complex.

An oligonucleotide covering the potential NFκB site of the IL-6 promoter competed against the induced binding to this fragment, while an oligonucleotide covering the NF-IL6 site was ineffective. When the NFκB oligonucleotide was used as probe, three IL-1/TNFα-induced complexes were observed.

Pretreatment with estradiol decreased the intensity of the slowest complex and strongly increased the intensity of the fastest migrating complex. The three bands were differentially supershifted (i.e., further decrease in the mobility of the complex due to binding of the antibody) by anti-p50 and anti-p65 antibodies, while none of several other antibodies tested, including anti-ER antibody, had any effect. Methylation interference assays showed identical DNA contact sites for all three complexes.

Ray, et al., J. Biol. Chem., 269(17):12940-946 (1994), not admitted to be prior art, describe that activation of the IL-6 promoter, elicited by a combination of NF-IL6 and the p65 subunit of NFκB, can be inhibited by the wt ER but not by an ER containing a mutation in its DNA binding domain. Furthermore, the repression of the IL-6 promoter by a combination ER and 17β-estradiol did not appear to be mediated via high affinity binding of the receptor to the promoter.

These data suggest that negative regulation by estrogen is mediated through the IL-6 promoter and is estrogen receptor dependent. Inhibition of IL-6 expression by estrogen is mediated through control of the transcriptional activity of NFκB or closely related proteins on the IL-6 promoter.

Mukaida, et al., J. Biol. Chem., 269(18):13289-295 (1994), not admitted to be prior art, describe that a glucocorticoid, dexamethasone, inhibited IL-8 production

at the transcriptional level. Mutation of either the AP-1 or NF-IL6 binding site on the IL-8 promoter did not abolish IL-8 gene repression by dexamethasone, suggesting that these sites were not targets for dexamethasone. Yet dexamethasone diminished the IL-1 induced formation of NFκB complexes.

The invention will now be described in greater detail by reference to the following non-limiting examples regarding the regulation of interleukin 6 transcription by estrogen receptor.

Examples

A candidate agent will be screened by either A) direct evaluation of protein binding to rel-sites, or B) indirect evaluation of binding to rel-sites.

A) Direct evaluation of protein binding to rel-sites

Cells selected for expression of the necessary components will be treated with the agent or vehicle control and an inducer (e.g., phorbol ester, cytokines, lipopolysaccharides). Cellular extracts prepared from those cells (e.g., whole cell, cytosolic, or nuclear extracts) will be analyzed for their DNA-binding using cytokine promoter fragments or various rel-sites as probes. Binding will be analyzed qualitatively (i.e., comparing pattern) and quantitatively comparing extracts from cells treated with vehicle or the agent.

B) Indirect evaluation of binding to rel-sites by

1) Measuring endogenous cytokine expression.

Cells selected for expression of the necessary components and their production of cytokine will be treated with the agent or vehicle control and an inducer (phorbol ester, cytokines, lipopolysaccharides). Activity of the agent will be quantitatively assessed by

measuring of cytokine using standard assays known to those skilled in the art.

2) Measuring the expression of a reporter introduced into the cell.

By means of transfection a reporter construct will be introduced into the cells that expresses an easily measurable protein under the control of a cytokine promoter or fragments thereof or isolated rel-sites. The other necessary components are either expressed endogenously by the cells or provided by cotransfection of expression vectors for the particular component. Cells will be treated with the agent or vehicle control and an effector (phorbol ester, cytokines, lipopolysaccharides). The activity of the agent will be analyzed quantitatively by measuring the expression of the reporter protein.

Agents will also be tested for their binding to IRs by traditional binding assays as well as for their activity to effect the classical mechanism of gene regulation by IRs. An agent that binds to IRs and regulates binding of rel proteins to cytokine promoters but does not activate the classical mechanism of IR action is a potential drug candidate for the specific treatment of diseases associated with aberrant expression of cytokines.

Experimental procedures employed in the examples described herein are set forth below:

Transient transfections and mammalian expression constructs

Construction of the pERE-tk-Luc reporter plasmid and the vector expressing ER_{gly} (pRShER) has been described (Tzukerman, M., A. Esty, D. Santiso-Mere, P. Danielian, M.G. Parker, R.B. Stein, J.W. Pike, and D.P. McDonnell (1994) "Human estrogen receptor transactivational capacity is determined by both cellular and promoter

context and mediated by two functionally distinct intramolecular regions," Mol. Endocrinol. 8:21-30, incorporated by reference herein). The pIL6[-225]Luc reporter construct was derived from the parental pIL6[-1200]Luc by excision of a NheI-BamHI fragment and religation of the vector fragment after blunt ending with Klenow DNA-polymerase. The parental pIL6[-1200]Luc was constructed by cloning the 1.2 kb IL-6 promoter insert excised with BamHI and KpnI from pCAT-M54-IL6(-) into the corresponding sites of the luciferase vector Lucpl.

C3H10T1/2 cells were seeded in phenol-red-free DMEM supplemented with 10% FBS at 80,000 cells per well (12-well plates). The cells were transfected by calcium phosphate precipitation (Peterson, J.L. and O.W. McBride (1980) "Cotransfer of linked eukaryotic genes and efficient transfer of hypoxanthine phosphoribosyltransferase by DNA-mediated gene transfer," Proc. Natl. Acad. Sci. U.S.A. 77:1583-1587) with 0.5 mg pIL6[-225]Luc alone or together with 0.05 mg pRShER or 0.1 mg HEO using pGEM as carrier to adjust to 2 mg total DNA in the transfection mix. After 4 h at 37 °C the cells were treated with 7% DMSO for 30 min followed by a medium change and addition of hormones. The following day the cells were induced with TNFα and IL-1b (1 nM each) for 24 h. After a brief wash with PBS the cells were lysed in 200 ml lysis buffer (25 mM Tris [pH 7.8], 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100). To 20 ml of each extract 100 ml of reagent (20 mM Tricine [pH 7.8], 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 279 mM coenzyme A, 470 mM luciferin, 530 mM ATP) was added and luciferase activity was measured immediately with a Dynatech luminometer in cycle mode.

Antibodies, IL-6 ELISA, and ER assay

Peptides used to raise the following antibodies in rabbits correspond to amino acid residues 91-105 of murine c-jun (Ryder and Nathans (1988) "Induction of protooncogene c-jun by serum growth factors," Proc. Natl. Acad. Sci. USA 85:8464-8467), 278-296 of murine NF-IL6 (Chang, C.J., T.T. Chen, H.Y. Lei, D.S. Chen, and S.C. Lee (1990) "Molecular cloning of a transcription factor, AGP/EBP, that belongs to members of the C/EBP family," Mol. Cell Biol. 10:6642-6653), 152-176 of murine c-rel (Bull, P., K.L. Morley, M.F. Hoekstra, T. Hunter, and I.M. Verma (1990) "The mouse c-rel protein has an N-terminal regulatory domain and a C-terminal transcriptional transactivation domain," Mol. Cell Biol. 10:5473-5485; Inoue, J., L.D. Kerr, L.J. Ransone, E. Bengal, T. Hunter, and I.M. Verma (1991) "c-rel activates but v-rel suppresses transcription from kappa B sites," Proc. Natl. Acad. Sci. U.S.A. 88:3715-3719), 347-361 of murine p50 (Ghosh, S., A.M. Gifford, L.R. Riviere, P. Tempst, G.P. Nolan, and D. Baltimore (1990) "Cloning of the p50 DNA binding subunit of NF-kappa B: homology to rel and dorsal," Cell 62:1019-1029), and 3-19 of human p65 (88% homology with murine p65) (Nolan, G.P., S. Ghosh, H.C. Liou, P. Tempst, and D. Baltimore (1991) "DNA binding and I kappa B inhibition of the cloned p65 subunit of NF-kappa B, a rel-related polypeptide," Cell 64:961-969; Ruben, S.M., P.J. Dillon, R. Schreck, T. Henkel, C.H. Chen, M. Maher, P.A. Baeuerle, and C.A. Rosen (1991) "Isolation of a rel-related human cDNA that potentially encodes the 65-kD subunit of NF-kappa B [letter]," Science 254:11). All the references mentioned above are incorporated by reference herein. All antibodies listed above were obtained affinity purified at a concentration of 1 mg/ml from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-TBP was a protein-A purified serum preparation from

a rabbit immunized with the full length human recombinant protein and reported to react with TBP from mouse, rat, and human origin (Santa Cruz Biotechnology, Inc.). Anti-ER is a mouse monoclonal antibody (IgG2a) raised against a peptide corresponding to amino acid residues 8-22 of the murine ER. IL-6 concentration in tissue culture supernatants was determined by use of an IL-6 ELISA kit (Endogen, Inc., Boston, MA) using murine IL-6 as standard.

ER in +/+LDA11 cells was measured in whole cell extracts. After washing and counting, cells were homogenized in buffer containing 50 mM Tris [pH 7.5], 30% glycerol, 500 mM KCl, 1 mM EDTA, 1 mM PMSF, and 5 mM DTT. After 30 min on ice the homogenate was centrifuged (100,000 g, 4 °C, 1 h). The supernatant was taken as whole cell extract, adjusted to 0.5% CHAPS, and incubated with 5 nM [^{3}H]estradiol in the absence or presence of a 200-fold excess of DES overnight at 4 °C. After incubation with anti-ER antibody, the complexes formed were precipitated with protein-A sepharose (Pharmacia), washed three times with 10 mM Tris [pH 7.5]/0.5% CHAPS, and measured by liquid scintillation counting.

<u>Electrophoretic mobility shift assay (EMSA) and methylation interference assay</u>

DNA binding studies were carried out with nuclear extracts from +/+LDA11 cells, extracts from yeast expressing recombinant human ER_{gly}, and purified p50 and p49 proteins. +/+LDA11 cells were maintained under conditions as described (Girasole, G., R.L. Jilka, G. Passeri, S. Boswell, G. Boder, D.C. Williams, and S.C. Manolagas (1992) "17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens,"

J. Clin. Invest. 89:883-891). To prepare nuclear extracts the cells were seeded in phenol-red-free McCoy's medium supplemented with 10% FBS and pretreated with hormone for 24 h if not indicated otherwise. After adjusting the medium to 2% FBS, the cells were induced with TNFα and IL-1b (1 nM each) for varying periods. In cases where cycloheximide (10 mg/ml) or the kinase inhibitor H7 (50 mM) were included, those compounds were added 5 min before induction. Incubation was stopped by two washes with ice cold PBS and cells were lysed in situ in cold buffer A (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2% Nonidet P-40). Lysates were transferred into microfuge tubes, nuclei pelleted (8000 rpm, 1 min) and resuspended in buffer C (20 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 420 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF). After 40 min rocking at 4 °C, samples were centrifuged (15,000 rpm, 10 min) and supernatants taken as nuclear extracts. Bradford protein assays (Bradford, M.M. (1976) "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," Anal. Biochem. 72:248-254) showed only minimal variations in protein concentrations which did not correlate with hormone or cytokine treatment. Extracts of yeast recombinantly expressing ER_{gly} were prepared from the BJ2168 strain transformed with YEpE10 as described (Tzukerman, M., A. Esty, D. Santiso-Mere, P. Danielian, M.G. Parker, R.B. Stein, J.W. Pike, and D.P. McDonnell (1994) "Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions," Mol. Endocrinol. 8:21-30). Purified, *Escherichia coli* expressed human p50 and p49 proteins were purchased from Promega (Madison, WI).

For EMSA, 2ml of the extracts were preincubated with 2 mg poly[dI-dC] in binding buffer adjusted to 20 mM HEPES [pH 7.9], 40 mM NaCl, 20 mM KCl, 2.5 mM $MgCl_2$, 10% glycerol, 0.1 mg/ml BSA, and 1 mM DTT. When the -225 to -52 IL-6 promoter fragment was used as probe 0.5 mg of Bluescript plasmid (Stratagene, La Jolla, CA) was also included. After 20 min on ice, the probe was added and the incubation continued for 20 min at room temperature. When antibodies were included, 1 mg was added 20 min after the probe and the incubation continued for 40 min at 4 °C. The complexes formed were analyzed on non-denaturing polyacrylamide gels (4% acrylamide/0.05% BIS; 2x200 mm) at 4 °C and 15 V/cm in 0.25xTBE. Probes were either double stranded oligonucleotides corresponding to the regions -82 to -47 (ATCAAATGTGGGATTTTCCCATGAGTCTCAATATTA) and -172 to -131 (CTAAAGGACGTCACATTGCACAATCTTAATAAGGTTTCCAAT) of the human IL-6 promoter and to the ERE of the vitellogenin promoter (Tzukerman, M., X.K. Zhang, T. Hermann, K.N. Wills, G. Graupner, and M. Pfahl (1990) "The human estrogen receptor has transcriptional activator and repressor functions in the absence of ligand," New Biol. 2:613-620) or the -225 to -52 NheI-SspI IL-6 promoter fragment. All probes were either labeled with [γ^{32}P]ATP using T4-polynucleotide kinase or with [α^{32}P]dATP using Klenow polymerase and subsequently purified by polyacrylamide gel electrophoresis.

For methylation interference assays, the -82 to -47 probe labeled with [γ^{32}P]ATP either on the upper or the lower strand was subjected to limited DMS-methylation (Maxam, A.M. and W. Gilbert (1980) "Sequencing end-labeled DNA with base-specific chemical cleavages," Methods Enzymol. 65:499-560). EMSA was performed as described above, scaled up 10-fold. Gels were blotted onto NA45 anion exchange membranes (Schleicher & Schuell) in 0.5xTBE for 30 min at 30 V (Singh, H., J.H.

LeBowitz, A.S. Baldwin, Jr., and P.A. Sharp (1988) "Molecular cloning of an enhancer binding protein: isolation by screening of an expression library with a recognition site DNA," Cell 52:415-423). After autoradiography, the DNA corresponding to the various complexes and the unretarded probe was eluted (10 min at 65 °C in 20 mM Tris [pH 8.0], 1 M NaCl, 0.1 mM EDTA) and purified by phenol/chloroform extraction and ethanol precipitation. After strand cleavage in 1 M piperidine (30 min at 90 °C) the fragments were resolved on denaturing polyacrylamide gels (12% acrylamide/0.6% BIS).

Example 1. Screening for ER mediated inhibition of IL-6 promoter activity

It has been shown that IL-6 repression is regulated by estradiol at the mRNA level (Girasole, G., R.L. Jilka, G. Passeri, S. Boswell, G. Boder, D.C. Williams, and S.C. Manolagas (1992) "17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens," J. Clin. Invest. 89:883-891; Jacobs, A.L., P.B. Sehgal, J. Julian, and D.D. Carson (1992) "Secretion and hormonal regulation of interleukin-6 production by mouse uterine stromal and polarized epithelial cells cultured in vitro," Endocrinology 131:1037-1046). To determine if estrogen or a candidate agent acts directly on IL-6 transcription, we transfected a reporter construct, expressing the firefly luciferase under the control of the human IL-6 promoter region from -225 to +14, into the murine fibroblast cell line C3H10T1/2. These cells can be considered as pre-osteoblasts since they differentiate into osteogenic cells in response to bone morphogenic protein-2 (Katagiri, T., A. Yamaguchi, T. Ikeda, S. Yoshiki, J.M. Wozney, V. Rosen, E.A. Wang, H.

Tanaka, S. Omura, and T. Suda (1990) "The non-osteogenic mouse pluripotent cell line, C3H10T1/2, is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic protein-2," Biochem. Biophys. Res. Commun. 172:295-299).

Therefore, C3H10T1/2 cells were transfected with a luciferase expression vector under the control of the proximal human IL-6 promoter (pIL6[-225]Luc) alone or together with the expression vector for the wild-type human ER_{gly} (pRShER). After pretreatment with varying concentrations of estradiol for 24 hours, the cultures were induced with 1 nM each of TNFα and IL-1 or left uninduced and 24 h later cells were harvested and extracts analyzed for luciferase activity.

Treatment of transfected cells with IL-1 and TNFα induced a 5-fold increase in luciferase activity over basal levels. Without cotransfection of a plasmid expressing the estrogen receptor, treatment with estradiol had no effect. However, with the expression of estrogen receptor by cotransfection, treatment with estradiol resulted in a strong, dose-dependent repression of luciferase activity.

Repression was observed with the wild-type human ER (ER_{gly}) as well as with an ER variant containing a glycine to valine point mutation in the hormone binding domain (ER_{val}) (Tora, L., A. Mullick, D. Metzger, M. Ponglikitmongkol, I. Park, and P. Chambon (1989) "The cloned human oestrogen receptor contains a mutation which alters its hormone binding properties," EMBO J. 8:1981-1986). While ER_{val} required a higher estradiol concentration, it exhibited a stronger repression. This is consistent with the finding that in induction experiments ER_{gly} responds at lower hormone concentrations but has considerable basal activity (Tzukerman, M., X.K. Zhang, T. Hermann, K.N. Wills, G. Graupner, and M. Pfahl (1990) "The human estrogen receptor has transcriptional

activator and repressor functions in the absence of ligand," New Biol. 2:613-620).

The dependence of the estrogen effect on cotransfected ER suggested that C3H10T1/2 cells do not express functional endogenous ER. This was confirmed by transfecting the cell with a luciferase reporter under the control of the vitellogenin estrogen response element (ERE) (Klein-Hitpass, L., M. Schorpp, U. Wagner, and G.U. Ryffel (1986) "An estrogen-responsive element derived from the 5′ flanking region of the Xenopus vitellogenin A2 gene functions in transfected human cells," Cell 46:1053-1061).

Therefore, C3H10T1/2 cells were transfected with a luciferase expression vector under the control of the minimal thymidine kinase promoter and the vitellogenin estrogen response element (pERE-tk-Luc) alone or together with pRShER. 24 h after treatment with 10 nM estradiol or vehicle cells were harvested and extracts analyzed for luciferase activity. Induction of luciferase activity by estradiol was only observed in the presence of cotransfected ER.

In addition, C3H10T1/2 cells were incubated with or without 10 nM estradiol. After 24 h the cultures were induced with TNFα and IL-1 (1 nM each) or left uninduced for additional 24 h. Il-6 in the supernatants was assayed by an ELISA specific for murine IL-6. C3H10T1/2 cells responded to IL-1 and TNFα treatment with strongly increased production of endogenous IL-6, but unlike other osteogenic or stromal cells containing endogenous ER (Girasole, G., R.L. Jilka, G. Passeri, S. Boswell, G. Boder, D.C. Williams, and S.C. Manolagas (1992) "17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens," J. Clin. Invest. 89:883-891), IL-6 levels were not decreased by estradiol. These data suggest

that the inhibition of IL-6 expression is at the transcriptional level and mediated through the ER.

By cotransfection studies using the preosteoblastic cell line C3H10T1/2, we showed that IL-1/TNFα-induced activation of the proximal IL-6 promoter region could be inhibited by estrogen. This inhibition was estrogen receptor dependent and was observed with both the wild-type human ER (ER_{gly}) and the ER_{val} variant. Similar results have been obtained by others in both HeLa cells cotransfected with ER_{val}, and in MBA13 cells, a preosteoblastic cell line expressing endogenous ER. Together with the described effects of estrogen on IL-6 mRNA (Girasole, G., R.L. Jilka, G. Passeri, S. Boswell, G. Boder, D.C. Williams, and S.C. Manolagas (1992) "17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens," J. Clin. Invest. 89:883-891; Jacobs, A.L., P.B. Sehgal, J. Julian, and D.D. Carson (1992) "Secretion and hormonal regulation of interleukin-6 production by mouse uterine stromal and polarized epithelial cells cultured in vitro," Endocrinology 131:1037-1046), these results suggest a transcriptional mechanism of estrogen-induced inhibition.

A candidate agent can be screened using the above assay, replacing estradiol with said agent.

Example 2. Screening agents that modulates binding of NFκB related proteins to the proximal IL-6 promoter

A cell line that expresses ER (+/+LDA11)

An exemplary assay system is a cell line that expressed all the necessary components endogenously, including the ER. The bone marrow derived murine stromal cell line +/+LDA11 has been shown to respond to IL-1 and TNFα treatment with strongly increased

secretion of IL-6. Treatment with estradiol inhibits this induction of IL-6 as shown for the protein and its mRNA (Girasole, G., R.L. Jilka, G. Passeri, S. Boswell, G. Boder, D.C. Williams, and S.C. Manolagas (1992) "17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens," J. Clin. Invest. 89:883-891).

To verify that ER is actually present in +/+LDA11 cells, hormone binding studies were carried out. Initial experiments showed a low number of specific estradiol binding sites in high salt extracts from these cells. Using a monoclonal antibody directed against the amino terminus of the ER, specifically bound [^{3}H]estradiol was immunoprecipitated confirming that the binding sites represented ER. From those studies we calculated that +/+LDA11 cells contain approximately 1000 ER molecules per cell.

However, when using electrophoretic mobility shift assays (EMSA) in combination with the vitellogenin ERE as a probe, ER-specific DNA binding activity could not be detected in nuclear extracts from +/+LDA11 treated with estradiol and/or IL-1 and TNFα. Nuclear extracts of +/+LDA11 cells pretreated with estradiol (10 nM) and TNFα and IL-1 (1nM each for 40 min) as indicated or yeast extract containing recombinantly expressed human wild-type ER_{gly} were incubated with the vitellogenin ERE as probe in the absence or presence of anti-ER antibody. Complexes formed were analyzed by EMSA.

The complexes detected are unrelated to the ER since they were not significantly affected by anti-ER antibody. Controls using ER containing extracts obtained from a yeast expression system gave rise to two slowly migrating complexes that were specifically shifted with the anti-ER antibody. These data suggest

that ER is present in +/+LDA11 cells but at concentrations too low to be detected by EMSA.

DNA-binding activity of nuclear extracts to the IL-6 promoter

To study the molecular mechanism of IL-6 induction, and its repression by estrogen, nuclear extracts from +/+LDA11 cells were analyzed for DNA-binding activity to the IL-6 promoter region that mediated cytokine induction and estrogen suppression in the cotransfection experiments. Since this DNA fragment showed a high background binding with nuclear extracts the most proximal region containing the TATA box was removed leaving a fragment from -225 to -52 upstream of the transcriptional start site. This region of the promoter contains consensus binding sites for several transcription factors including a core sequence of the cAMP response element (CRE), a binding site for the leucine zipper protein NF-IL6, and a NFκB site (Isshiki, H., S. Akira, O. Tanabe, T. Nakajima, T. Shimamoto, T. Hirano, and T. Kishimoto (1990) "Constitutive and interleukin-1 (IL-1)-inducible factors interact with the IL-1-responsive element in the IL-6 gene," Mol. Cell Biol. 10:2757-2764).

Binding of NF-IL6 and NFκB-like proteins to these sites has been demonstrated (Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto (1990) "A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family," EMBO J. 9:1897-1906; Libermann, T.A. and D. Baltimore (1990) "Activation of interleukin-6 gene expression through the NF-kappa B transcription factor," Mol. Cell Biol. 10:2327-2334). This fragment was incubated with nuclear extracts from +/+LDA11 cells that had been treated with IL-1 and TNFα for various times. +/+LDA11 cells were pretreated with 10 nM estradiol as indicated.

After 24 h the cells were induced with TNFα and IL-1 (1 nM each) for various periods of time. Induction was stopped by cell lysis and nuclear extracts were analyzed by EMSA using the -225 to -52 IL-6 promoter fragment as probe (Figure 1a).

Complexes formed were analyzed by EMSA. After treatment with the cytokines an inducible complex was observed. The intensity of the complex was maximal already after 10 min treatment with IL-1 and TNFα and decreased gradually over time. After 2 hours of induction the intensity of the complex was significantly reduced.

Pretreatment of the cells with estradiol had no effect on the binding capacity of extracts from uninduced cells. However, estradiol pretreatment resulted in a marked increase of the induced complex with induction intervals from 10 min to 40 min but only a slight effect on the complex after 2 h of induction. In addition to the increased intensity, pretreatment with estradiol also caused a qualitative change, increasing the mobility of the complex.

<u>Detecting the composition of the DNA-binding complex</u>

To investigate the nature of the complex and the proteins potentially involved, we incubated the binding reactions with antibodies directed against several potential binding factors. Nuclear extract from +/+LDA11 cells treated with estradiol (10 nM) and TNFα and IL-1 (1nM each for 10 min) as indicated were incubated with the -225 to -52 probe in the absence or presence of various antibodies. Complexes formed were analyzed by EMSA.

Fig. 1b shows that none of the antibodies tested affected DNA binding of extracts from uninduced cells. Neither anti-c-jun, nor anti-c-rel, nor anti-NF-IL6

antibodies had any effect on the cytokine induced complexes.

However, anti-p50 and anti-p65, antibodies directed against the two proteins in the NFκB complex, abolished formation of the complex (lanes 10-13). This was observed with extracts derived from cells treated or untreated with estradiol, over the whole period of cytokine induction (Fig. 1b depicts the results at 10 minutes after the induction). With longer exposures, a very weak complex of low mobility was detected, probably resulting from a supershift of the induced complex by anti-p50 and anti-p65.

Although ER binding activity to the vitellogenin ERE was not detectable in +/+LDA11 extracts, we tested whether the ER was involved in complex formation on the IL-6 promoter fragment. Nuclear extracts of +/+LDA11 cells treated with estradiol (10 nM) and TNFα and IL-1 (1nM each for 40 min) as indicated or yeast extract containing recombinantly expressed human wild-type ER were incubated with the -225 to -52 probe in the absence or presence of anti-ER antibody. Complexes formed were analyzed by EMSA.

Fig. 1c shows that independent of cytokine induction or estradiol treatment, addition of the anti-ER antibody did not significantly affect any of the complexes, induced or constitutive. In addition, when yeast extracts containing high concentrations of recombinant ER were incubated with the IL-6 promoter fragment no specific binding of the ER was detected (lanes 7 and 8). The weak bands observed are unrelated to the ER, since they were not affected by the anti-ER antibody.

The results from the antibody gel shift experiments were further supported by oligonucleotide competition studies. Nuclear extract from +/+LDA11 cells treated with estradiol (10 nM) and TNFα and IL-1 (1nM each for 10 min) as indicated were incubated with the -225 to -52

probe in the absence or presence of a 400-fold molar excess of oligonucleotides corresponding to the regions of -82 to -47 and -172 to -131 of the human IL-6 promoter. Complexes formed were analyzed by EMSA.

The arrows in Fig. 1d indicate the complexes formed upon induction with TNFα and IL-1. Inclusion of an oligonucleotide covering the NF-IL6 site, the CRE, and an adjacent CCAAT-box of the IL-6 promoter (-172 to -131) in the binding reaction in 400-fold excess over the labeled -225 to -52 fragment did not affect any of the complexes, constitutive or cytokine-induced (lanes 4 and 7). However, an oligonucleotide covering the putative NFκB site and adjacent sequences (-82 to -47) specifically abolished the formation of the cytokine induced complexes (lanes 3 and 6).

The antibody experiments and the oligonucleotide competition studies suggested that IL-1 and TNFα specifically activated NFκB or related proteins. No binding of c-jun (AP-1), NF-IL6, c-rel, or ER was detected.

The lack of NF-IL6 binding is surprising, since induction and binding of this transcription factor in response to IL-1 has been reported for other cells (Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto (1990) "A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family," <u>EMBO J.</u> 9:1897-1906; Inoue, J., L.D. Kerr, L.J. Ransone, E. Bengal, T. Hunter, and I.M. Verma (1991) "c-rel activates but v-rel suppresses transcription from kappa B sites," <u>Proc. Natl. Acad. Sci. USA</u> 88:3715-3719). Our DNA binding experiments show that in the bone marrow derived +/+LDA11 cell IL-1 and TNFα induce the binding of NFκB or closely related proteins to the IL-6 promoter. Similar results have been obtained in different cell types (H. Shimizu, K. Mitomo, T. Watanabe, S. Okamoto,

and K. Yamamoto (1990) "Involvement of a NF-kappa B-like transcription factor in the activation of the interleukin-6 gene by inflammatory lymphokines," Mol. Cell Biol. 10:561-568; Zhang, Y.H., J.X. Lin, and J. Vilcek (1990) "Interleukin-6 induction by tumor necrosis factor and interleukin-1 in human fibroblasts involves activation of a nuclear factor binding to a kappa B-like sequence," Mol. Cell Biol. 10:3818-3823). Neither induced nor uninduced binding of several other factors with potential binding sites in the proximal IL-6 promoter fragment could be detected, including AP-1. This transcription factor is one of the paradigms for direct inhibition by intracellular receptor including GR, RAR and TR. The mechanism of AP-1 inhibition can involve protein-protein interaction and/or competition for DNA binding depending on the particular gene (Diamond, M.I., J.N. Miner, S.K. Yoshinaga, and K.R. Yamamoto (1990) "Transcription factor interactions: selectors of positive or negative regulation from a single DNA element," Science 249:1266-1272; Schüle, R., K. Umesono, D.J. Mangelsdorf, J. Bolado, J.W. Pike, and R.M. Evans (1990) "Jun-Fos and receptors for vitamins A and D recognize a common response element in the human osteocalcin gene," Cell 61:497-504; Yang-Yen, H.F., J.C. Chambard, Y.L. Sun, T. Smeal, T.J. Schmidt, J. Drouin, and M. Karin (1990) "Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction," Cell 62:1205-1215; Yang-Yen, H.F., X.K. Zhang, G. Graupner, M. Tzukerman, B. Sakamoto, M. Karin, and M. Pfahl (1991) "Antagonism between retinoic acid receptors and AP-1: implications for tumor promotion and inflammation," New Biol. 3:1206-1219; Zhang, X.K., K.N. Wills, M. Husmann, T. Hermann, and M. Pfahl (1991) "Novel pathway for thyroid hormone receptor action through interaction with jun and fos oncogene

activities," Mol. Cell Biol. 11:6016-6025). Several reports also suggest a cross-talk between ER and AP-1, however there is no evidence for estrogen dependent inhibition of AP-1 activity (Gaub, M.P., M. Bellard, I. Scheuer, P. Chambon, and P. Sassone-Corsi (1990) "Activation of the ovalbumin gene by the estrogen receptor involves the fos-jun complex," Cell 63:1267-1276; Tzukerman, M., X.K. Zhang, and M. Pfahl (1991) "Inhibition of estrogen receptor activity by the tumor promoter 12-O-tetradeconylphorbol-13-acetate: a molecular analysis," Mol. Endocrinol. 5:1983-1992). Taken together, it is highly unlikely that AP-1 plays a role in the negative regulation of IL-6 expression by estrogen.

Example 3. Screening agents that differentially affect distinct complexes with the IL-6 promoter

Distinctive complexes with the IL-6 promoter

Treatment of +/+LDA11 cells with IL-1 and TNFα induced binding of NFκB or related proteins to the IL-6 promoter. Since pretreatment with estradiol not only increased the intensity but also the mobility of the complexes, we investigated the binding of +/+LDA11 nuclear extracts to the oligonucleotide covering the putative NFκB site (-82 to -47).

+/+LDA11 cells were pretreated with 10 nM estradiol as indicated. After 24 h the cells were induced with TNFα and IL-1 (1 nM each) for various periods of time. Induction was stopped by cell lysis and nuclear extracts were analyzed by EMSA using the -82 to -47 IL-6 promoter fragment as probe. Fig. 2a shows that extracts from cells treated with IL-1 and TNFα exhibited 3 induced complexes (A,B,C) when compared with extracts from untreated cells.

Over the course of induction (10-120 min) in particular the fastest migrating complex (C) decreased in intensity. Interestingly, estradiol pretreatment reduced the intensity of the slowest migrating complex (A) while strongly increasing the intensity of the fastest band (C). This corresponds to the pattern obtained with the -225 to -52 fragment where complexes seemed to migrate faster with estradiol treatment (Fig. 3).

It is likely that with both fragments analogous complexes were formed; however, only with the shorter oligonucleotide were they completely resolved. Nuclear extract from +/+LDA11 cells treated with estradiol (10 nM) and TNFα and IL-1 (1nM each for 40 min) as indicated were incubated with the -82 to -47 probe in the absence or presence of a 100-fold molar excess of oligonucleotides corresponding to the regions of -82 to -47 and -172 to -131 of the human IL-6 promoter or the vitellogenin ERE. Complexes formed were analyzed by EMSA.

All three induced complexes (A,B,C) were specific since their formation was abolished by inclusion of a 100-fold excess of the unlabeled probe (Fig. 2b, lanes 6 and 10), while the same molar excess of the NF-IL6 oligonucleotide (-172 to -131) or the vitellogenin ERE had no effect (Fig. 2b, lanes 7, 8, 11, and 12).

Fig. 2c shows that when binding of the extracts to the oligonucleotide covering the NF-IL6 site (-172 to -131) was investigated, several complexes were detected. Nuclear extract from +/+LDA11 cells treated with estradiol (10 nM) and TNFα and IL-1 (1nM each for 40 min) as indicated were incubated with the -172 to -131 probe in the absence or presence of a 100-fold molar excess the unlabeled oligonucleotide. Complexes formed were analyzed by EMSA. The arrows in Fig. 2c indicate

the complexes A, B, and C formed upon induction with TNFα and IL-1.

Two of the complexes were specific, since they could be competed with an excess of the unlabeled oligonucleotide (lanes 2, 4, and 6). However, all of the complexes were formed constitutively, independent of cytokine induction or estradiol treatment, suggesting that they were unrelated to the regulation of IL-6 expression by IL-1, TNFα, and estrogen.

Screening for compounds that affect the formation of distinct complexes

In more detailed studies we analyzed the effects of other compounds on the formation of complexes A, B, and C (Fig. 3). +/+LDA11 cells were pretreated with cycloheximide (CHX) or the kinase inhibitor H7 for 5 min or with estradiol (10 nM) and/or ICI 164,384 (100 nM) for 24 h or 60 min before induction with TNFα and IL-1 (1nM each for 30 min). Treatment was stopped by cell lysis and nuclear extracts were analyzed by EMSA using the -82 to -47 fragment as probe. Arrows indicate the induced complexes A, B, and C.

Pretreatment of the cells with the protein synthesis inhibitor cycloheximide before addition of cytokines did not interfere with complex formation (lane 8). This is consistent with the fast induction of binding and has been shown before for the activation of NFκB (Henkel, T., T. Machleidt, I. Alkalay, M. Kronke, Y. Ben-Neriah, and P.A. Baeuerle (1993) "Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B," Nature 365:182-185; Sen, R. and D. Baltimore (1986) "Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism," Cell 47:921-928). It has been reported that cycloheximide treatment activates NFκB binding (Sen, R. and D. Baltimore (1986)

"Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism," Cell 47:921-928; Zhang, Y.H., J.X. Lin, and J. Vilcek (1990) "Interleukin-6 induction by tumor necrosis factor and interleukin-1 in human fibroblasts involves activation of a nuclear factor binding to a kappa B-like sequence," Mol. Cell Biol. 10:3818-3823). However, in +/+LDA11 cells we did not observe any induction by cycloheximide (lane 7). Additionally, pretreatment with H-7, a potent protein kinase C (PKC) inhibitor (Kawamoto, S. and H. Hidaka (1984) "1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H-7) is a selective inhibitor of protein kinase C in rabbit platelets," Biochem. Biophys. Res. Commun. 125:258-264), did not interfere with induction of complex formation by IL-1 and TNFα (lane 10). This suggests that induction of complexes A, B, and C is mediated through a PKC-independent pathway and is consistent with the finding that IL-1 and TNFα activate NFκB and induce IL-6 independently of PKC (11,55,99).

Activation of NFκB by phorbol esters is probably mediated by PKC (Baeuerle, P.A. and D. Baltimore (1988) "I kappa B: a specific inhibitor of the NF-kappa B transcription factor," Science 242:540-546). However, treatment of +/+LDA11 cells with 12-O-tetradeconylphorbol-13-acetate (TPA) did not significantly induce production of IL-6, nor did it induce complex formation with the -82 to -47 fragment.

The estradiol effect, i.e. decreasing complex A and increasing complex C (Fig. 3, compare lanes 2 and 3), was not seen with a short estradiol pretreatment (60 min) before induction (lane 6). In addition, the pure anti-estrogen ICI 164,384 (Wakeling, A.E. and J. Bowler (1988) "Biology and mode of action of pure antioestrogens," J. Steroid Biochem. 30:141-147) did not affect the complex pattern (lane 4). However, when ICI

164,384 was added in combination with estradiol it prevented the effect mediated by the estrogen (lane 5).

Since ICI 164,384 acts as an antagonist via binding to the ER, these results further support the hypothesis that the effects of estradiol on the induced complexes are receptor mediated. However, the mechanism of estrogen action is probably indirect, as indicated by the lack of response to short term estradiol treatment.

<u>Screening for agents that affect binding characteristics of the proteins in distinct complexes</u>

To investigate the binding characteristics of the proteins in complexes A, B, and C with the NFκB oligonucleotide, methylation interference experiments were carried out (Fig. 4). Nuclear +/+LDA11 extracts from cells induced with TNFα and IL-1 (1nM each) were incubated with -82 to -47 probe that had been labeled either on the upper or the lower strand and subjected to limited DMS-methylation. After preparative EMSA, DNA from complexes A, B, and C and from the unretarded probe (F) was isolated, cleaved with piperidine, and electrophoresed on a 12% denaturing gel. The sequence corresponding to the NFκB consensus site is shown boxed, a cryptic AP-1 site is shaded.

On both strands N-7-methylation of the guanine bases within the NFκB site (-73 to -63, boxed) interfered with complex formation, while methylation of guanines flanking the consensus site had no observable effect. The interference pattern for all three complexes (A,B,C) was identical.

The observation that methylation of guanines just outside of the NFκB site (-75, -60, -58) did not affect the formation of even the largest complex (A) suggests that in all three complexes DNA contacts are made within the same core region. In addition, the lack of DNA binding interference with methylation of guanines -60, -

58, and -56 strongly argues against any cytokine induced binding of factors to the nonconsensus (TGAGTCT, shaded) AP-1 site (Tanabe, O., S. Akira, T. Kamiya, G.G. Wong, T. Hirano, and T. Kishimoto (1988) "Genomic structure of the murine IL-6 gene. High degree conservation of potential regulatory sequences between mouse and human," J. Immunol. 141:3875-3881) in this region (-61 to -55).

Our studies show that estrogen affects the formation of complexes with the IL-6 promoter that involve NFκB p50 and p65 or very closely related proteins. Treatment of +/+LDA11 cells with IL-1 and TNFα specifically induced the formation of at least three distinct complexes with the NFκB consensus site in the IL-6 promoter. Although of various size, in all three complexes the DNA contacts are restricted to the core sequence of the NFκB site. The corresponding core sequence of other NFκB elements is protected by p50 and p65 (Baldwin, A.S., Jr. and P.A. Sharp (1988) "Two transcription factors, NF-kappa B and H2TF1, interact with a single regulatory sequence in the class I major histocompatibility complex promoter," Proc. Natl. Acad. Sci. USA 85:723-727; Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Bail, M.B. Urban, P. Kourilsky, P.A. Baeuerle, and A. Israel (1990) "The DNA binding subunit of NF-kappa B is identical to factor KBF1 and homologous to the rel oncogene product," Cell 62:1007-1018; Sen, R. and D. Baltimore (1986) "Multiple nuclear factors interact with the immunoglobulin enhancer sequences," Cell 46:705-716), both of which have been shown to directly interact with DNA (Nolan, G. P., S. Ghosh, H.C. Liou, P. Tempst, and D. Baltimore (1991) "DNA binding and I kappa B inhibition of the cloned p65 subunit of NF-kappa B, a rel-related polypeptide," Cell 64:961-969; Urban, M.B., R. Schreck, and P.A. Baeuerle (1991) "NF-kappa B contacts DNA by a heterodimer of the p50 and p65 subunit," EMBO J.

10:1817-1825). C-rel homodimers and heterodimers with p50 have been shown to bind the NFκB site in the IL-6 promoter (Nakayama, K., H. Shimizu, K. Mitomo, T. Watanabe, S. Okamoto, and K. Yamamoto (1992) "A lymphoid cell-specific nuclear factor containing c-Rel-like proteins preferentially interacts with interleukin-6 kappa B-related motifs whose activities are repressed in lymphoid cells," Mol. Cell Biol. 12:1736-1746).

In addition, this study showed that in lymphoid cells c-rel or an immunologically related factor is a component of a larger complex that binds the NFκB site in the IL-6 promoter and functions as a constitutive repressor. In +/+LDA11 cells, we could not detect any c-rel specific binding activity. A number of other NFκB unrelated proteins have been shown to bind to NFκB consensus sites. Those include aA-CRYBP1 (Nakamura, T., D.M. Donovan, K. Hamada, C.M. Sax, B. Norman, J.R. Flanagan, K. Ozato, H. Westphal, and J. Piatigorsky (1990) "Regulation of the mouse alpha A-crystallin gene: isolation of a cDNA encoding a protein that binds to a cis sequence motif shared with the major histocompatibility complex class I gene and other genes," Mol. Cell Biol. 10:3700-3708), MBP-1/PRDII-BFI (Baldwin, A.S., Jr., K.P. LeClair, H. Singh, and P.A. Sharp (1990) "A large protein containing zinc finger domains binds to related sequence elements in the enhancers of the class I major histocompatibility complex and kappa immunoglobulin genes," Mol. Cell Biol. 10:1406-1414; Fan, C.M. and T. Maniatis (1990) "A DNA-binding protein containing two widely separated zinc finger motifs that recognize the same DNA sequence," Genes Dev. 4:29-42), and AGIE-BP1 (Ron, D., A.R. Brasier, and J.F. Habener (1991) "Angiotensinogen gene-inducible enhancer-binding protein 1, a member of a new family of large nuclear proteins that recognize nuclear

factor kappa B-binding sites through a zinc finger motif," Mol. Cell Biol. 11:2887-2895).

It has been shown that C/EBP-like proteins attenuate NFκB mediated transactivation of the angiotensinogen gene acute-phase response element (Brasier, A.R., D. Ron, J.E. Tate, and J.F. Habener (1990) "A family of constitutive C/EBP-like DNA binding proteins attenuate the IL-1 alpha induced, NF kappa B mediated trans-activation of the angiotensinogen gene acute-phase response element," EMBO J. 9:3933-3944). Currently, we cannot exclude that those proteins or others are part of the observed complexes or are involved in the inhibition of IL-6 expression by estrogen. A recent study suggested that in uterine cells, estradiol induced complex formation with an NFκB element (Shyamala, G. and M.C. Guiot (1992) "Activation of kappa B-specific proteins by estradiol," Proc. Natl. Acad. Sci. USA 89:10628-10632). The induced complex did not contain p50 or p65 and therefore may represent other factors.

Example 4. Screening for agents that affects the binding of p65 to IL-6 promoter

Analyzing composition of the complexes formed with the NFκB site

As with the larger promoter fragment, we analyzed the nature of the complexes formed with the NFκB oligonucleotide (-82 to -47) by antibody shift experiments (Fig. 5a). Nuclear extract from +/+LDA11 cells treated with estradiol (10 nM) and TNFα and IL-1 (1nM each for 10 min) as indicated were incubated with the -82 to -47 probe in the absence or presence of various antibodies. Complexes formed were analyzed by EMSA.

We observed strong effects on the induced complexes when anti-p50 or anti-p65 (lanes 17-20) were included in

the binding reactions. Interestingly, anti-p50 specifically abolished the formation of complexes B and C, seemed to leave complex A unaffected, and caused the appearance of a single supershifted band (S1). Anti-p65, however, inhibited the formation of all three induced complexes and produced two supershifted bands (S1, S2). This suggested that p65 or an immunologically closely related protein is part of all three induced complexes, while p50 or a related protein is only present in complexes B and C.

It has been reported that recombinantly expressed c-rel binds to the NFκB site in the IL-6 promoter as heterodimer with p50 and, with particular high affinity, as homodimer (Nakayama, K., H. Shimizu, K. Mitomo, T. Watanabe, S. Okamoto, and K. Yamamoto (1992) "A lymphoid cell-specific nuclear factor containing c-Rel-like proteins preferentially interacts with interleukin-6 kappa B-related motifs whose activities are repressed in lymphoid cells," Mol. Cell Biol. 12:1736-1746). When anti-c-rel was included in the binding reactions with the +/+LDA11 extracts the antibody did not inhibit any of the induced complexes. On longer exposures a weak supershifted complex was detectable. This complex migrated at the same position as the supershift observed with anti-p50 suggesting that it did not contain the larger c-rel protein. Since the peptide used to raise the anti-c-rel antibody has a 56% homology to the analogous p50 sequence (Ghosh, S., A.M. Gifford, L.R. Riviere, P. Tempst, G.P. Nolan, and D. Baltimore (1990) "Cloning of the p50 DNA binding subunit of NF-kappa B: homology to rel and dorsal," Cell 62:1019-1029; Inoue, J., L.D. Kerr, L.J. Ransone, E. Bengal, T. Hunter, and I.M. Verma (1991) "c-rel activates but v-rel suppresses transcription from kappa B sites," Proc. Natl. Acad. Sci. USA 88:3715-3719), it is likely that this weak band is the result of a cross-reactivity and unrelated to c-

rel. As with the larger promoter fragment, anti-c-jun did not affect complex formation (lanes 13 and 14). Results shown depict the 10 min induction time point and are essentially identical with longer cytokine treatments.

None of the antibodies tested had a marked effect on complex formation with the uninduced extracts. Only anti-p50 produced a very weak supershifted complex migrating at the S1 position as observed with the induced extracts (only visible on longer exposures). This weak binding activity, only detectable when supershifted, could either result from a cytosolic contamination or represent basal activation under the culture conditions.

In additional EMSA experiments we included a purified preparation of recombinant p50 as well as the yeast expressed ER (Fig 5b). Nuclear +/+LDA11 extracts from cells pretreated with estradiol (10 nM) and induced with TNFα and IL-1 (1nM each for 30 min) as indicated as well as purified human p50 protein and yeast extract containing recombinantly expressed human ER were incubated with -82 to -47 probe in the absence or presence of various antibodies. Complexes formed were analyzed by EMSA. Arrows indicate complexes A, B, and C induced by cytokine treatment and the complexes S1 and S2 resulting from the antibody supershifts.

As expected, neither did yeast expressed ER bind to the NFκB -82 to -47 fragment (lanes 5, 9, 13, and 17) nor was ER involved in the formation of the induced complexes as indicated by the lack of any anti-ER antibody effect (lanes 14-17). Purified p50 bound to this fragment and was specifically supershifted by anti-p50 but not by anti-p65 (compare lanes 4, 8, and 12).

Surprisingly, the complex formed with purified recombinant p50 migrated slower than complexes B and C. Using lower concentrations of purified p50 did not

affect the migration, suggesting that the band represented p50 homodimers and not higher order complexes (Duckett, C.S., N.D. Perkins, T.F. Kowalik, R.M. Schmid, E.S. Huang, A.S. Baldwin, Jr., and G.J. Nabel (1993) "Dimerization of NF-KB2 with RelA(p65) regulates DNA binding, transcriptional activation, and inhibition by an I kappa B-alpha (MAD-3)," Mol. Cell Biol. 13:1315-1322). However, the antibody shift experiments suggested that both NFκB proteins, p50 and p65, are part of complexes B and C (lanes 6, 7, 11, and 12) and consequently both complexes should migrate slower than p50 homodimers (Urban, M.B., R. Schreck, and P.A. Baeuerle (1991) "NF-kappa B contacts DNA by a heterodimer of the p50 and p65 subunit," EMBO J. 10:1817-1825).

It is possible that the proteins in complexes B and C are only immunologically related to p50 and p65 but actually of smaller size. Speculations that the p50 homologue p49 is part of the induced complexes and is responsible for the faster migration could not be confirmed. Although purified p49 bound the -82 to -47 IL-6 fragment and strongly cross-reacted with the anti-p50 antibody, the complex formed migrated even more slowly than the p50 complex. This corresponds to results obtained with other NFκB binding sites (Duckett, C.S., N.D. Perkins, T.F. Kowalik, R.M. Schmid, E.S. Huang, A.S. Baldwin, Jr., and G.J. Nabel (1993) "Dimerization of NF-KB2 with RelA(p65) regulates DNA binding, transcriptional activation, and inhibition by an I kappa B-alpha (MAD-3)," Mol. Cell Biol. 13:1315-1322).

However, the finding that the anti-p50 antibody strongly cross-reacts with p49 indicates that the antibodies used may detect other NFκB related proteins in the complexes. Alternatively, the migration of complexes B and C could be higher than the migration

observed with recombinant p50 due to conformational differences resulting from post-translational modification. This would correlate with the observation that the inclusion of anti-p50, abolishing complexes B and C, produced a supershifted complex (S1) migrating more slowly than the supershifted complex obtained with recombinant p50 (Fig. 5b, lanes 6-8).

A closer inspection of the band shift results indicated that anti-p50 also affected complex A. As discussed before, treatment with estradiol not only increased the intensity of complex C but also decreased the intensity of complex A (lanes 2 and 3). We consistently observed that inclusion of anti-p50 had a very similar effect: the antibody specifically decreased the intensity of complex A formed with the extracts from cells not treated with estradiol, resulting in equal intensity of this band using extracts from estradiol treated or untreated cells (Fig. 5b, compare lanes 2 and 7). These results suggest that band A induced by IL-1 and TNFα in the absence of estradiol is composed of two different unresolved complexes, one (A1) that is also induced in the presence of estradiol and does not contain p50, and another complex (A2) containing p50 (or an immunologically related protein).

Treatment with estradiol may increase the intensity of complex C at the expense of complex A2. If complex A2 represents a transcriptionally more active state, this could explain the inhibitory effect of estradiol on IL-6 expression. Recently it has been shown that the TATA binding protein (TBP or TFIIDt) directly interacts with NFκB (Kerr, L.D., L.J. Ransone, P. Wamsley, M.J. Schmitt, T.G. Boyer, Q. Zhou, A.J. Berk, and I.M. Verma (1993) "Association between proto-oncoprotein Rel and TATA-binding protein mediates transcriptional activation by NF-kappa B," Nature 365:412-419). Therefore, we were interested if TBP was involved in the formation of the

induced complexes. However, using an anti-TBP antibody we could not detect any participation of TBP in complexes A, B, or C.

Our antibody gel shift experiments suggested that p65 is a component of all three observed complexes. This particular protein is the NFκB component containing the transactivation domain (Schmitz, M.L. and P.A. Baeuerle (1991) "The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B," EMBO J. 10:3805-3817). Within the different complexes the transactivation function may be differentially active. The antibody shift experiments suggest that estradiol diminishes the A2 complex while increasing complex C. The slow migrating A2 complex may contain other factor(s) involved in the transactivation process. The TATA-binding protein TBP, part of the TFIID complex has been reported to interact strongly with c-rel and p65, but not with p50 or p49 (Kerr, L.D., L.J. Ransone, P. Wamsley, M.J. Schmitt, T.G. Boyer, Q. Zhou, A.J. Berk, and I.M. Verma (1993) "Association between proto-oncoprotein Rel and TATA-binding protein mediates transcriptional activation by NF-kappa B," Nature 365:412-419). However, using a TBP-specific antibody we could not detect TBP as part of any of the complexes formed with the NFκB site in the IL-6 promoter.

Example 5. Efficacy-testing of Putative Cytokine Modulators

Methods for testing the efficacy of putative cytokine modulators are provided. Each candidate compound is tested for its efficacy in modulating cytokine expression in cell lines, in animal models, and in controlled clinical studies using methods known to those skilled in the art and approved by the Food and Drug Administration, such as, but not limited to, those

promulgated in The Federal Register 47 (no. 56): 12558-12564, March 23, 1982.

Example 6. Toxicity-testing of Putative Cytokine Modulators

Methods are provided for determining whether an agent active in any of the methods listed above has little or no effect on healthy cells. Such agents are then formulated in a pharmaceutically acceptable buffer or in buffers useful for standard animal tests.

By "pharmaceutically acceptable buffer" is meant any buffer which can be used in a pharmaceutical composition prepared for storage and subsequent administration, which comprise a pharmaceutically effective amount of an agent as described herein in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A.R. Gennaro edit. 1985). Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. For example, sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid may be added as preservatives. Id. at 1449. In addition, antioxidants and suspending agents may be used. Id.

A. Additional screens for Toxicity: Method 1

Agents identified as having cytokine modulating activity are assessed for toxicity to cultured human cells. This assessment is based on the ability of living cells to reduce 2,3,-bis[2-methoxy-4-nitro-5-sulphonylphenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide] otherwise referred to as XTT (Paull et al., J. Heterocyl. Chem. 25:763-767 (1987); Weislow et al., (1989), J. Natl. Canc. Inst. 81:577). Viable mammalian cells are capable of reductive cleavage of an N-N bond

in the tetrazole ring of XTT to form XTT formazan. Dead cells or cells with impaired energy metabolism are incapable of this cleavage reaction. The extent of the cleavage is directly proportional to the number of living cells tested. Cells from a human cell line such as HeLa cells are seeded at 10^3 per well in 0.1 ml of cell culture medium (Dulbecco' s modified minimal essential medium supplemented with 10% fetal calf serum) in the wells of a 96 well microtiter plate. Cells are allowed to adhere to the plate by culture at 37° C in an atmosphere of 95% air, 5% CO_2. After overnight culture, solutions of test substances are added in duplicate to wells at concentrations that represent eight half-decade log dilutions. In parallel, the solvent used to dissolve the test substance is added in duplicate to other wells. The culture of the cells is continued for a period of time, typically 24 hours. At the end of that time, a solution of XTT and a coupler (methylphenazonium sulfate) is added to each of the test wells and the incubation is continued for an additional 4 hours before the optical density in each of the wells is determined at 450 nm in an automated plate reader. Substances that kill mammalian cells, or impair their energy metabolism, or slow their growth are detected by a reduction in the optical density at 450 nm in a well as compared to a well which received no test substance.

B. Additional screens for Toxicity: Method 2

Cytokine modulators are tested for cytotoxic effects on cultured human cell lines using incorporation of ^{35}S methionine into protein as an indicator of cell viability. HeLa cells are grown in 96 well plates in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum and 50μg/ml penicillin and streptomycin. Cells are initially seeded at 10^3 cells/well, 0.1 ml/well. Cells are grown for 48 hrs without exposure to the cytokine modulator, then medium

is removed and varying dilutions of the cytokine modulator prepared in complete medium are added to each well, with control wells receiving no cytokine modulator. Cells are incubated for an additional 48-72 hrs. Medium is changed every 24 hrs and replaced with fresh medium containing the same concentration of the cytokine modulators. Medium is then removed and replaced with complete medium without antifungal. Cells are incubated for 24 hr in the absence of cytokine modulator, then viability is estimated by the incorporation of ^{35}S into protein. Medium is removed, replaced with complete medium without methionine, and incubated for 30 min. Medium is again removed, and replaced with complete medium without methionine but containing 0.1 μCi/ml ^{35}S methionine. Cells are incubated for 3 hrs. Wells are washed 3 times in PBS, then cells are permeabilized by adding 100% methanol for 10 min. Ice cold 10% trichloroacetic acid (TCA) is added to fill wells; plates are incubated on ice for 5 min. This TCA wash is repeated two more times. Wells are again washed in methanol, then air dried. 50μl of scintillation cocktail are added to each well and dried onto the wells by centrifugation. Plates are used to expose X ray film. Densitometer scanning of the autoradiogram, including wells without antifungal, is used to determine the dosage at which 50% of cells are not viable (ID_{50}) (Culture of Animal Cells. A manual of basic technique. (1987). R. Ian Freshney. John Wiley & Sons, Inc., New York).

Example 7. Administration of Cytokine Modulators

The invention features novel cytokine modulators discovered by the methods described above. It also includes novel pharmaceutical compositions which include cytokine modulators discovered as described above formulated in pharmaceutically acceptable formulations.

Furthermore, the invention features a method for treating a subject inflicted with a pathological condition affected by the level of a cytokine by administering to that subject a therapeutically effective amount of a cytokine modulator. Such administration can be by any method known to those skilled in the art, for example, by topical application or by systemic administration.

By "therapeutically effective amount" is meant an amount that relieves (to some extent) one or more symptoms of the disease or condition in the patient. Additionally, by "therapeutically effective amount" is meant an amount that returns to normal, either partially or completely, physiological or biochemical parameters associated with or causative of a mycotic disease or condition. Generally, it is an amount between about 1 nmole and 1 μmole of the molecule, dependent on its EC_{50} and on the age, size, and disease associated with the patient.

Other embodiments of this invention are disclosed in the following claims.

WHAT IS CLAIMED IS

1. Method for screening for a therapeutic agent for treatment of a pathological condition affected by the level of a cytokine, comprising the steps of:

contacting a potential therapeutic agent with a system comprising an intracellular receptor, a promoter or a portion of said promoter with a rel site, and a protein that binds to said rel site on said promoter;

measuring the binding of said protein to said rel site on said promoter; wherein a reduction in the binding of said protein to the rel site on the promoter compared to the binding of said protein in the absence of said agent is an indication that said agent is potentially useful for treatment of said condition.

2. The method of claim 1, wherein said protein is a rel-like protein.

3. The method of claim 2, wherein said rel-like protein is NFκB.

4. The method of claim 1, wherein said system further comprises a ligand for said intracellular receptor.

5. The method of claim 1, wherein said condition is osteoporosis.

6. The method of claim 1, wherein said condition is rheumatoid arthritis.

7. The method of claim 1, wherein said condition is inflammation.

8. The method of claim 1, wherein said condition is psoriasis.

9. The method of claim 1, wherein said condition is Kaposi's sarcoma.

10. The method of claim 1, wherein said condition is septic shock.

11. The method of claim 1, wherein said condition is multiple myeloma.

12. The method of claim 1, wherein said intracellular receptor is a steroid receptor.

13. The method of claim 1, wherein said intracellular receptor is an estrogen receptor.

14. The method of claim 1, wherein said intracellular receptor is selected from the group consisting of retinoid acid receptors, retinoid X receptors, glucocorticoid receptor, progesterone receptors, androgen receptor, thyroid hormone receptors, and vitamin D receptor.

15. The method of claim 1, wherein said measuring comprises determining the expression level of a cytokine or an acute phase protein.

16. The method of claim 1, wherein said measuring comprises determining the expression level of a reporter gene linked to said promoter.

17. The method of claim 1, wherein said system further comprises an effector of said promoter.

18. The method of claim 17, wherein said effector is selected from the group consisting of tumor necrosis factor, interleukin-1, viruses, endotoxin, phorbol

esters, epidermal growth factor, leukemia inhibitor factor and cAMP agonists.

19. The method of claim 1, wherein said cytokine is interleukin 6.

20. The method of claim 1, wherein said cytokine is interleukin 8.

21. The method of claim 1, wherein said system is a cell.

22. The method of claim 1, wherein said system is an extract of a cell.

23. The method of claim 21, wherein said intracellular receptor is expressed from a transfected vector.

24. The method of claim 21, wherein said promoter or said portion of said promoter is transfected into said cell.

FIG. 1a.

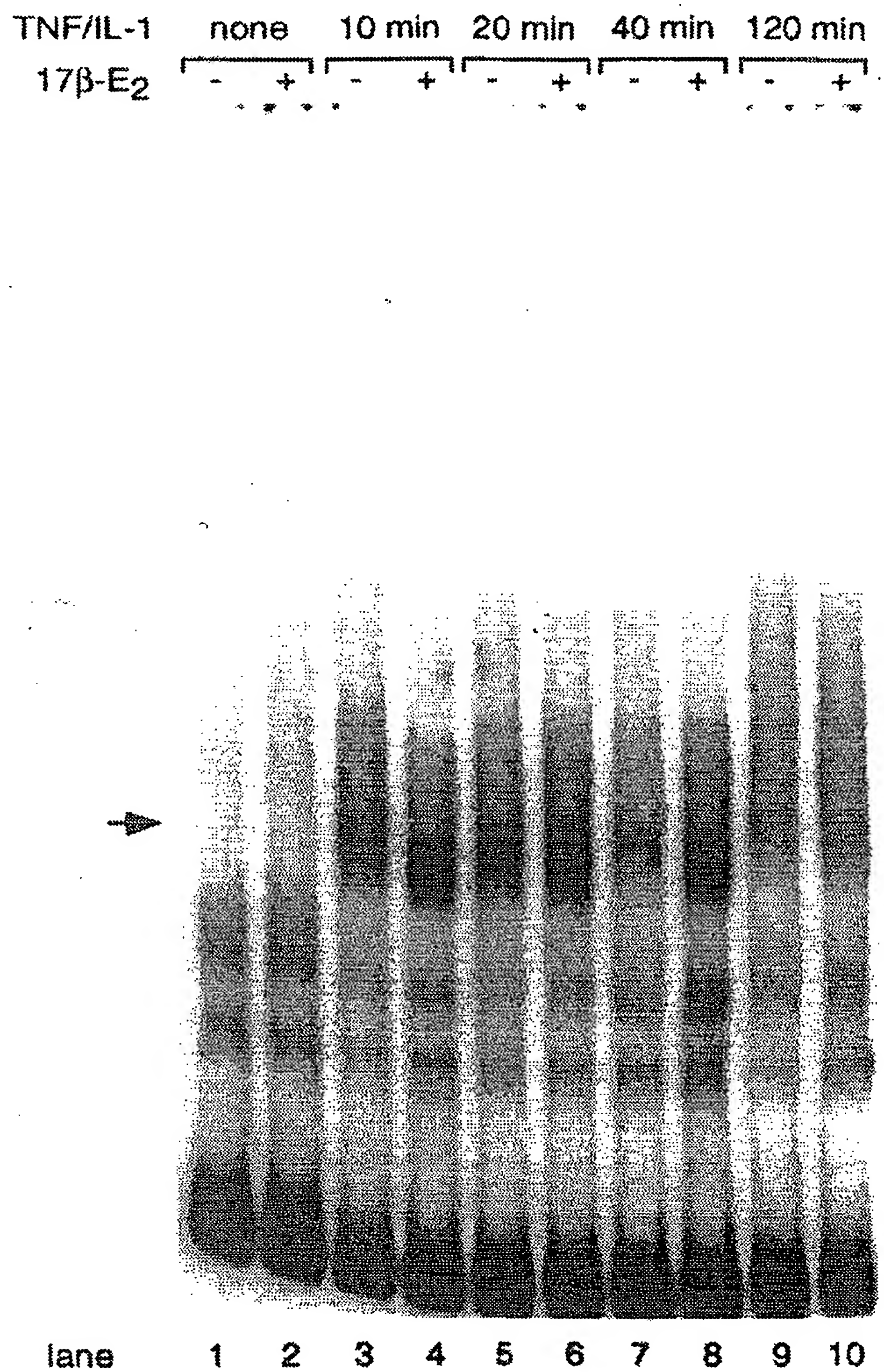

TNF/IL-1
none
10 min
20 min
40 min
120 min
17β-E2
- + - + - + - + - +
lane
1 2 3 4 5 6 7 8 9 10

FIG. 1b.

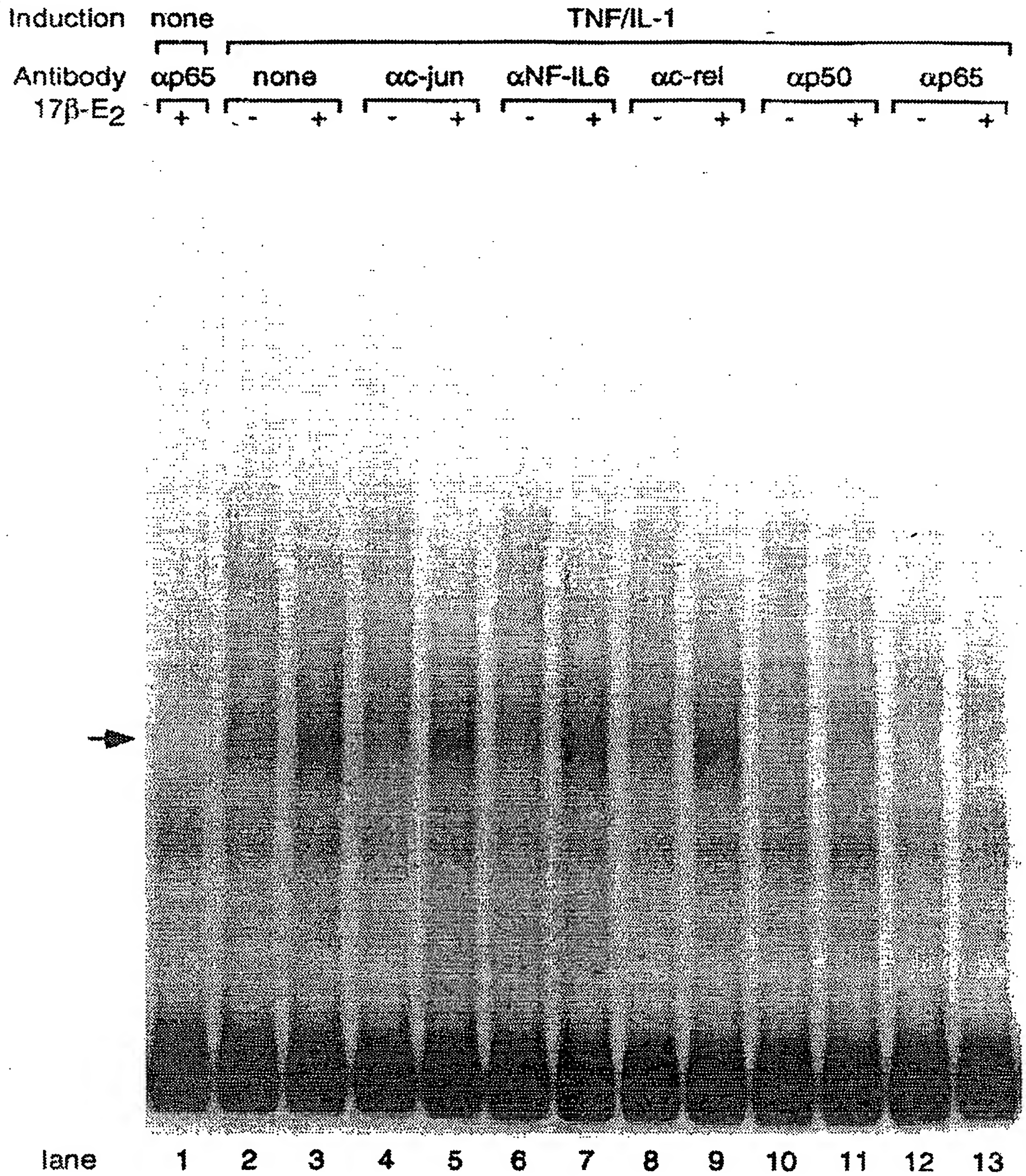
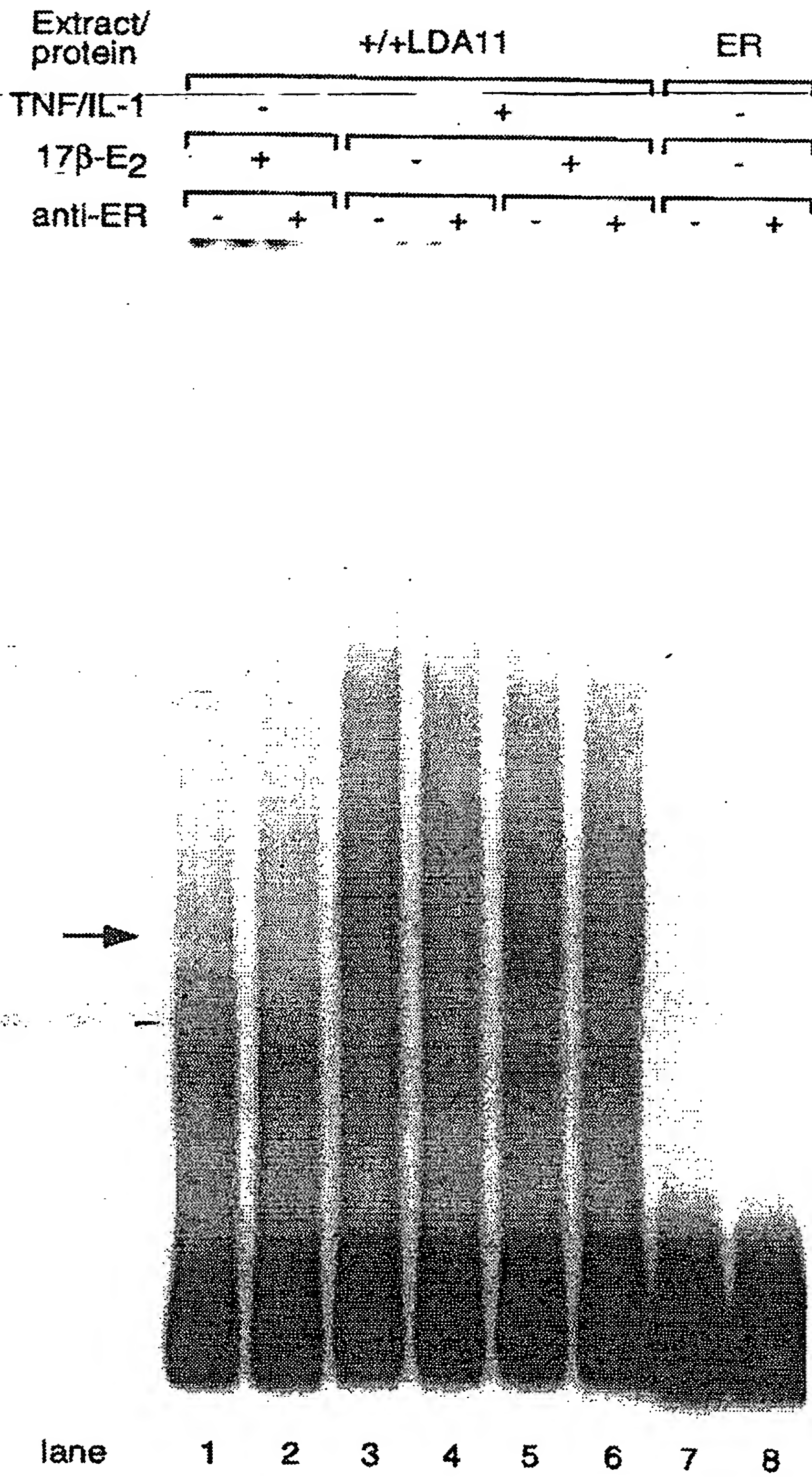

Induction
none
TNF/IL-1
Antibody
αp65
none
αc-jun
αNF-IL6
αc-rel
αp50
αp65
17β-E2
+
- +
- +
- +
- +
- +
- +
lane
1 2 3 4 5 6 7 8 9 10 11 12 13

FIG. 1c.


Extract/ protein
+/+LDA11
ER
TNF/IL-1
-
+
-
17β-E2
+
-
+
-
anti-ER
- + - + - + - +
lane 1 2 3 4 5 6 7 8

4/11

FIG. 1d.

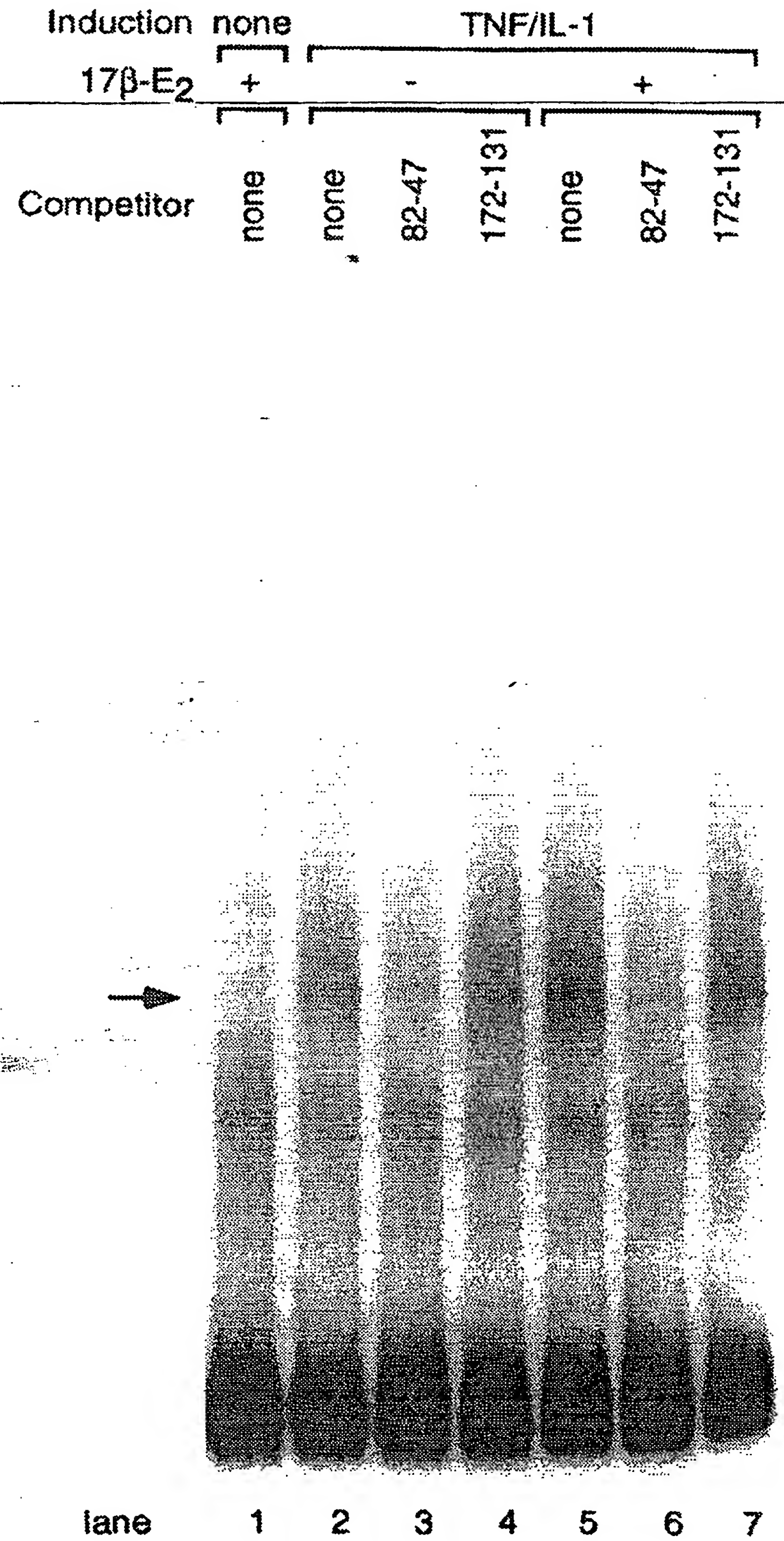

Induction none TNF/IL-1
17β-E2 + - +
Competitor none none 82-47 172-131 none 82-47 172-131
lane 1 2 3 4 5 6 7

FIG. 2a.

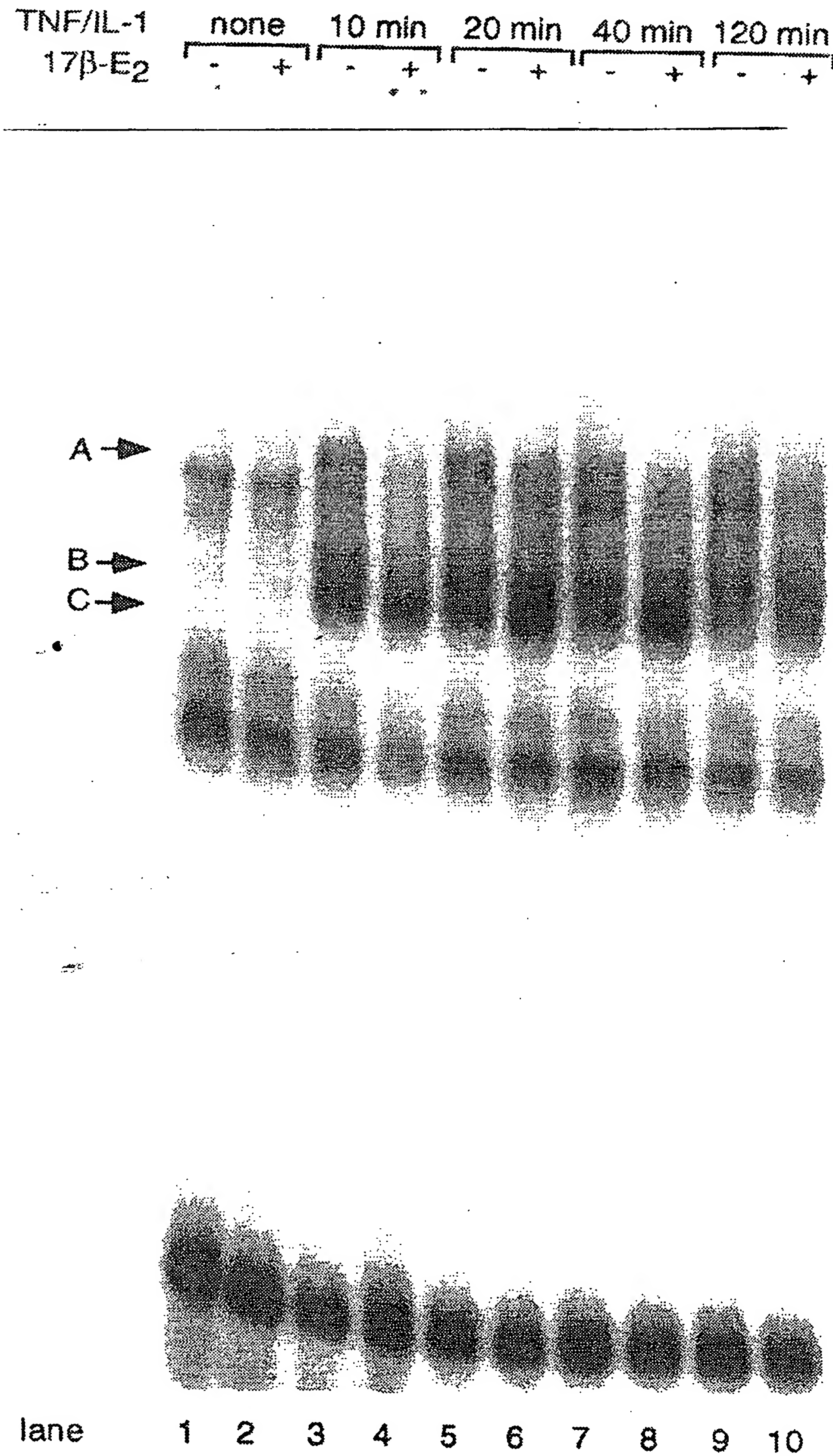

TNF/IL-1
none
10 min
20 min
40 min
120 min
17β-E2
- + - + - + - + - +
A
B
C
lane
1 2 3 4 5 6 7 8 9 10

FIG. 2b.

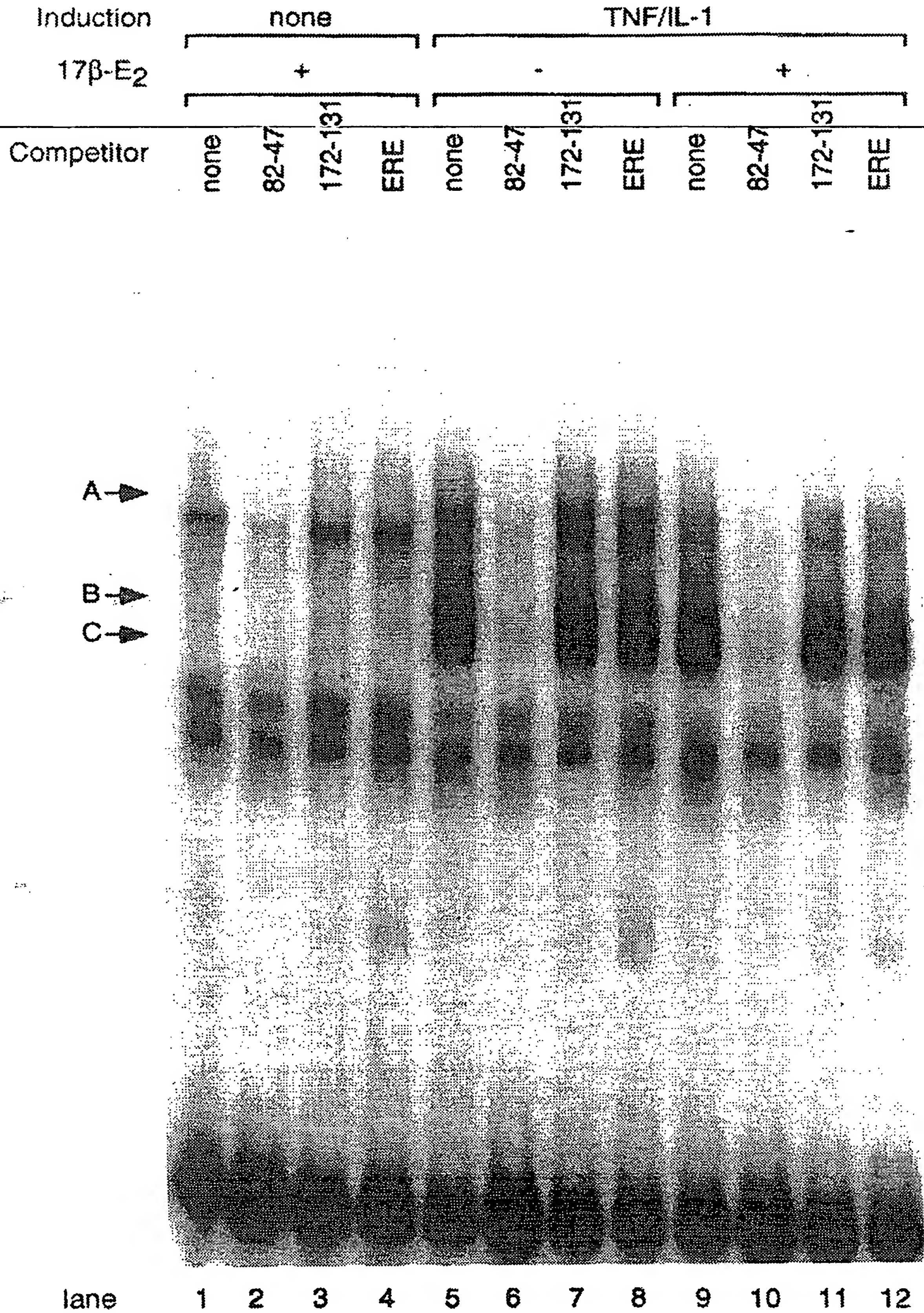

Induction
none
TNF/IL-1
17β-E2
+
-
+
Competitor
none
82-47
172-131
ERE
none
82-47
172-131
ERE
none
82-47
172-131
ERE
A
B
C
lane
1 2 3 4 5 6 7 8 9 10 11 12

FIG. 2c.

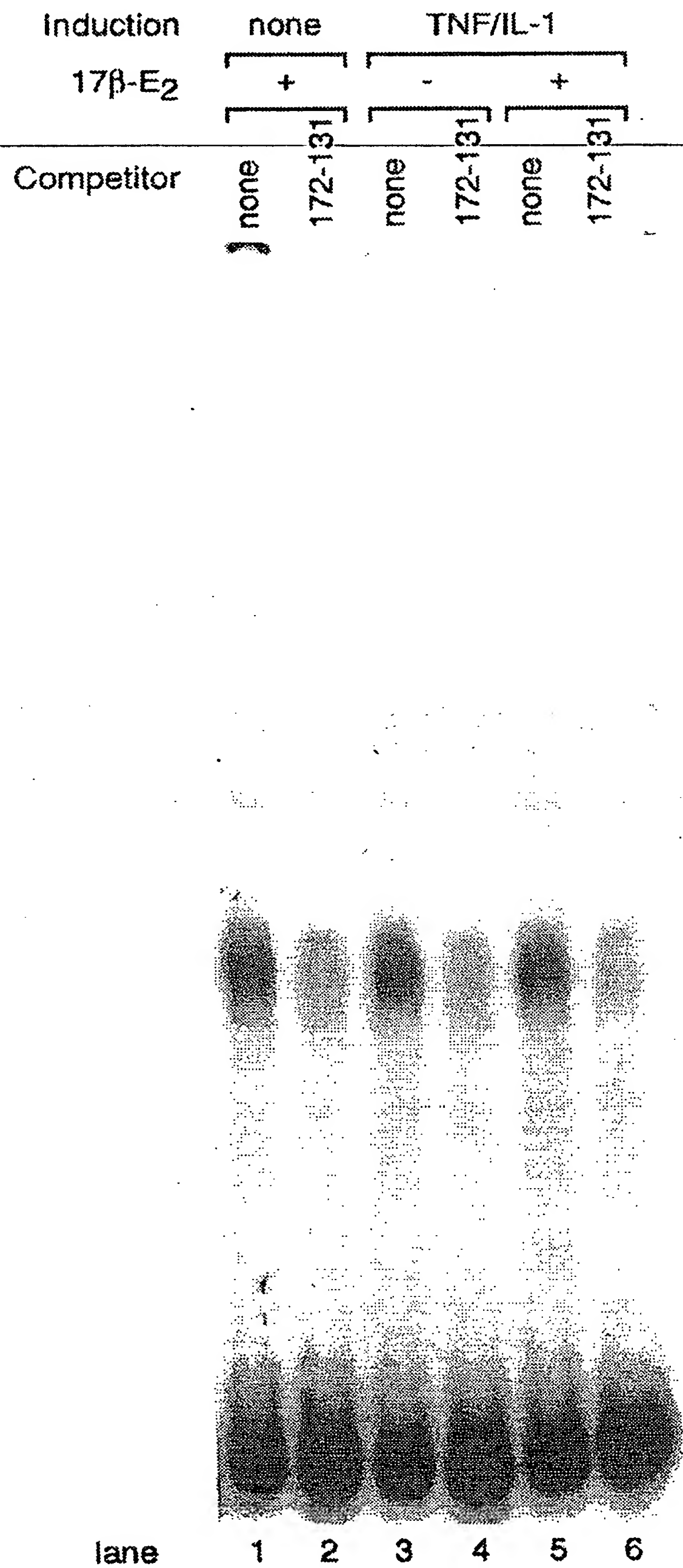

Induction
none
TNF/IL-1
17β-E2
+
-
+
Competitor
none
172-131
none
172-131
none
172-131
lane
1
2
3
4
5
6

FIG. 3.

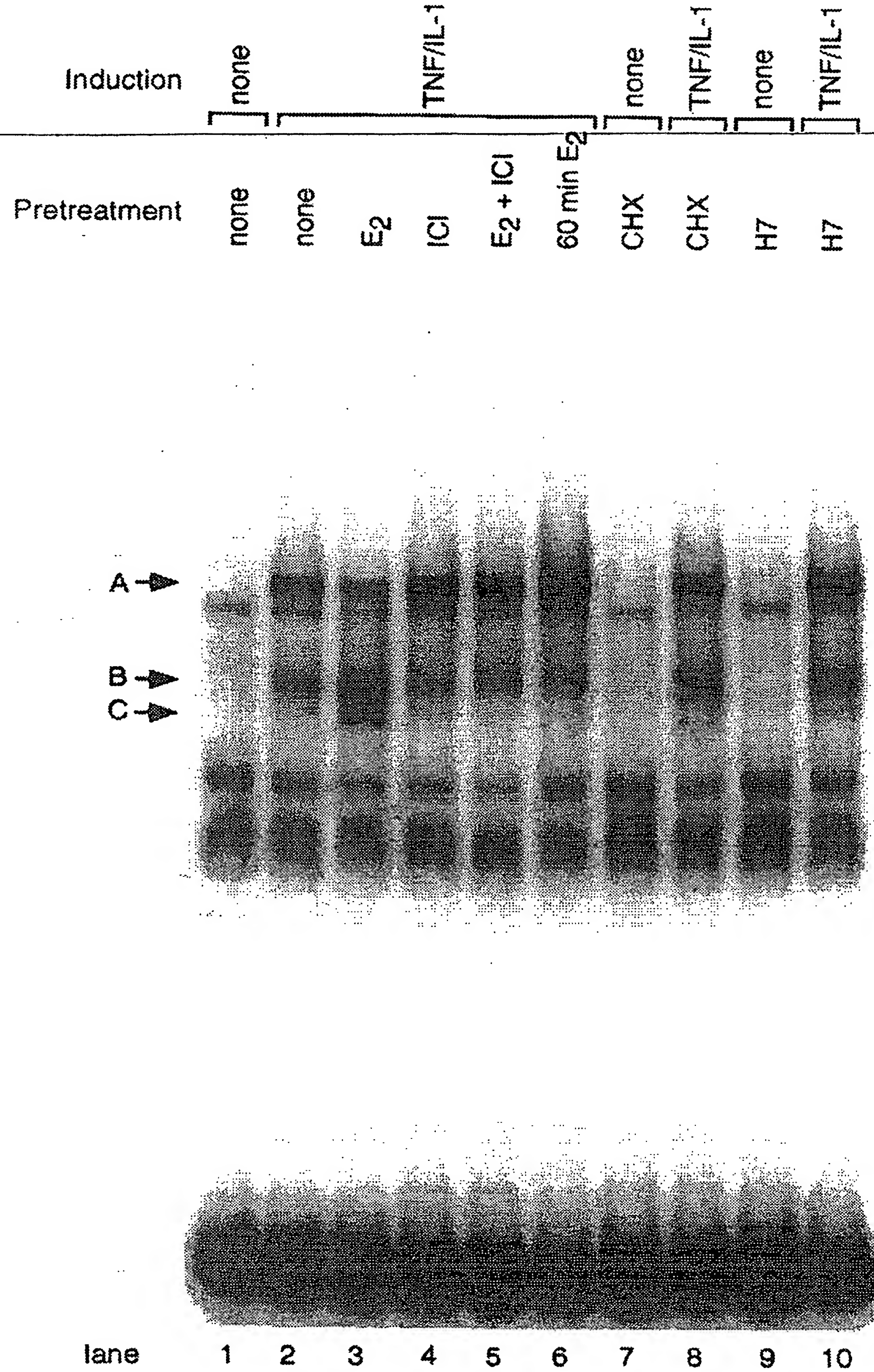

Induction
none
TNF/IL-1
none
TNF/IL-1
none
TNF/IL-1
Pretreatment
none
none
E2
ICI
E2 + ICI
60 min E2
CHX
CHX
H7
H7
A
B
C
lane
1
2
3
4
5
6
7
8
9
10

FIG. 4.

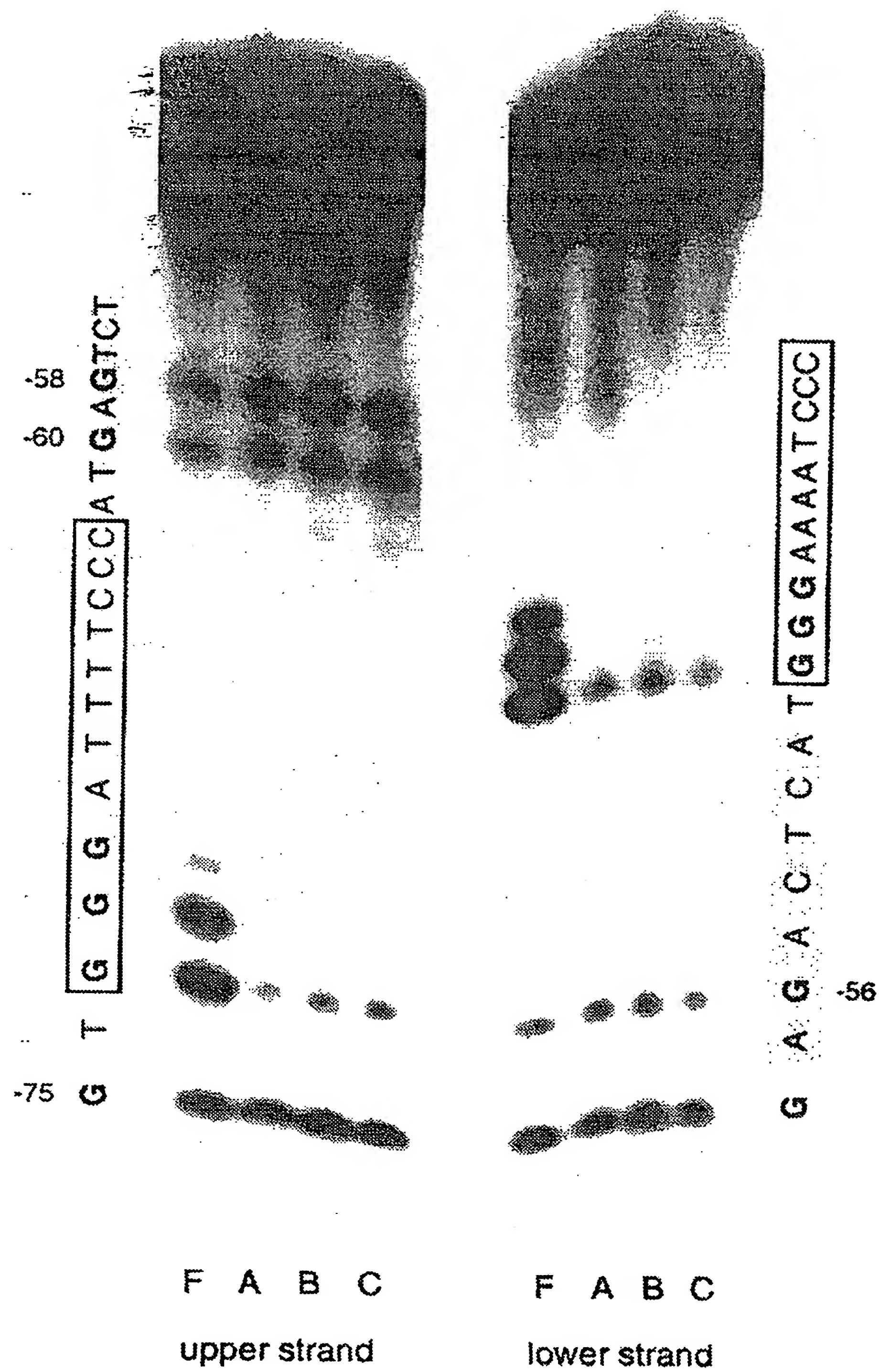

-58
-60
-75
G T G G A T T T C C C A T G A G T C T
G A G A C T C A T G G G A A A T C C C
-56
F A B C
upper strand
F A B C
lower strand

FIG. 5a.

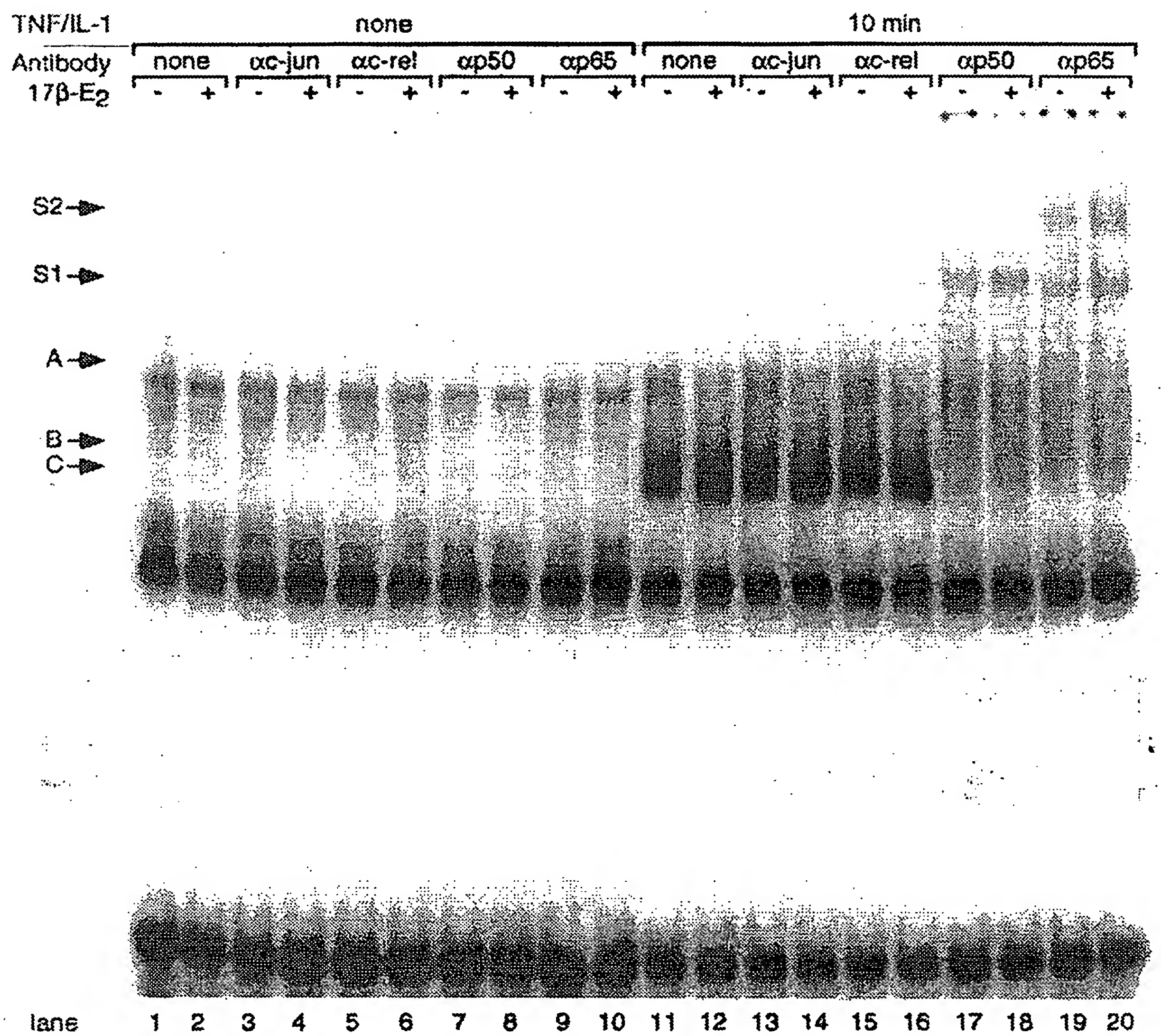
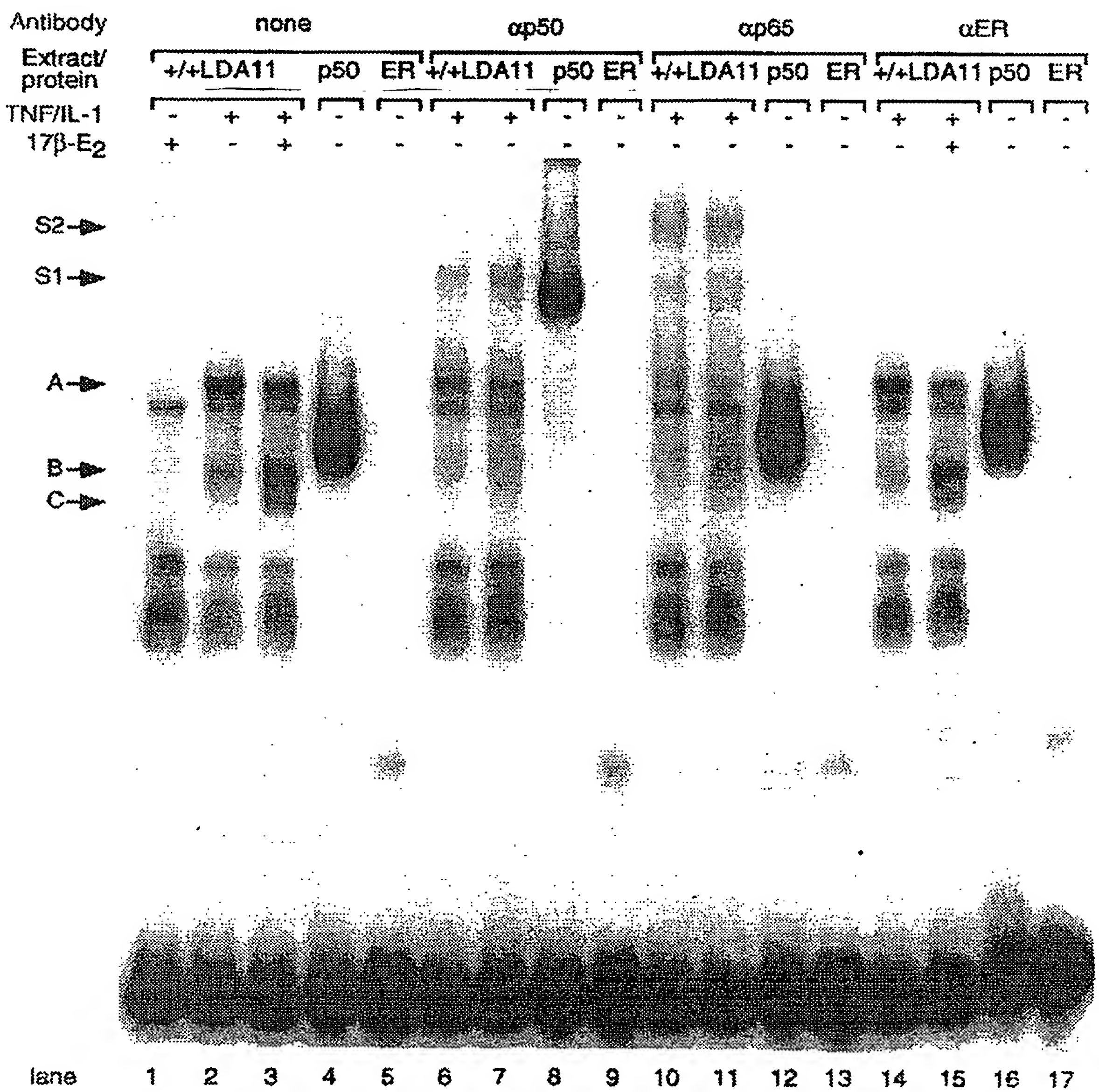

TNF/IL-1
none
10 min
Antibody
none
αc-jun
αc-rel
αp50
αp65
none
αc-jun
αc-rel
αp50
αp65
17β-E2
- + - + - + - + - + - + - + - + - + - +
S2
S1
A
B
C
lane
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

FIG. 5b.


Antibody
none
αp50
αp65
αER
Extract/ protein
+/+LDA11 p50 ER
+/+LDA11 p50 ER
+/+LDA11 p50 ER
+/+LDA11 p50 ER
TNF/IL-1
17β-E2
S2
S1
A
B
C
lane
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

INTERNATIONAL SEARCH REPORT

Inter   nal Application No
PCT/US 95/06524

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/50 G01N33/68

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,92 07072 (LA JOLLA CANCER RESEARCH FOUNDATION.THE REGENTS OF THE U. CALIFORNIA) 30 April 1992 cited in the application ---	
A	THE EMBO JOURNAL, vol. 9, no. 6, 1990 pages 1897-1906, S. AKIRA ET AL. 'A nuclear factor for IL-6 expression is a member of a C/EBP family.' cited in the application --- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. IMMUNOL., vol. 142, no. 9, 1 May 1989 pages 3134-3139, S. S. TABIBZADEH ET AL. 'Cytokine-induced production of IFN-B2/IL-6 by freshly explanted human endometrial stromal cells.' cited in the application	
P,A	MOLECULAR ENDOCRINOLOGY, vol. 9, no. 4, April 1995 pages 401-412, E. CALDENHOVEN ET AL. 'Negative cross-talk between RelA and the Glucocorticoid receptor: a possible mechanism for the antiinflamatory action of glucocorticoids. '	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 95/06524

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9207072	30-04-92	AU-A-	8946991	20-05-92
		CA-A-	2093811	11-04-92
		EP-A-	0552302	28-07-93
		JP-T-	6502532	24-03-94

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(54) Title: NUCLEOTIDE AND PROTEIN SEQUENCES OF LATS GENES AND METHODS BASED THEREON

(57) Abstract

The present invention relates to a tumor suppressor gene, termed large tumor suppressor (lats), and methods for identifying tumor suppressor genes. The method provides nucleotide sequences of *lats genes*, and amino acid sequences of their encoded proteins, as well as derivatives (e.g., fragments) and analogs thereof. In a specific embodiment, the lats protein is a human protein. The invention further relates to fragments (and derivatives and analogs thereof) of lats which comprise one or more domains of a lats protein. Antibodies to lats, its derivatives and analogs, are additionally provided. Methods of production of the lats proteins, derivatives and analogs, e.g., by recombinant means, are also provided. Therapeutic and diagnostic methods and pharmaceutical compositions are provided. The invention also relates to recombinant plants and animals and methods of increasing the growth of edible plants and animals. In specific examples, isolated lats genes, from *Drosophila*, mouse, and human, and the sequences thereof, are provided.

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NUCLEOTIDE AND PROTEIN SEQUENCES OF *LATS* GENES AND METHODS BASED THEREON

1. INTRODUCTION

This application is a continuation-in-part of copending application Serial No. 08/411,111 filed March 27, 1995, which is incorporated by reference herein in its entirety.

The present invention relates to tumor suppressor genes, in particular to "*lats*" genes (large tumor suppressor) and their encoded protein products, as well as derivatives and analogs thereof. Production of lats proteins, derivatives, and antibodies is also provided. The invention further relates to therapeutic compositions and methods of diagnosis and therapy.

2. BACKGROUND OF THE INVENTION

Tumorigenesis in humans is a complex process involving activation of oncogenes and inactivation of tumor suppressor genes (Bishop, 1991, Cell 64:235-248). Tumor suppressor genes in humans have been identified through studies of genetic changes occurring in cancer cells (Ponder, 1990, Trends Genet. 6:213-218; Weinberg, 1991, Science 254:1138-1146). In *Drosophila*, tumor suppressor genes have been previously identified by recessive overproliferation mutations that cause late larval and pupal lethality (Gateff, 1978, Science 200:1448-1459; Gateff and Mechler, 1989, CRC Crit. Rev. Oncogen 1:221-245; Bryant, 1993, Trends Cell Biol. 3:31-35; Török et al., 1993, Genetics 135:71-80). Mutations of interest were identified when dissection of dead larvae and pupae revealed certain overproliferated tissues. Several genes identified in homozygous mutants have been cloned including *l(1)discs large-1*(*dlg*; Woods and Bryant, 1991, Cell 66:451-464; Woods and Bryant, 1993, Mechanisms of Development 44:85-89), *fat* (Mahoney et al., 1991, Cell 67:853-868), *l(2)giant larvae* (*lgl*. Lützelschwab et al., 1987, EMBO J. 6:1791-1797; Jacob et al., 1987, Cell 50:215-225), *expanded* (*ex*; Boedigheimer and Laughon, 1993, Development

118:1291-1301; Boedigheim r et al., 1993, Mechanisms of D velopment 44:83-84), *hyperplastic discs* (*hyd*; Mansfield et al., 1994, Developmental Biology 165:507-526) and the gene encoding th S6 ribosomal protein (Watson et al., 1992, Proc. Natl. Acad. Sci. USA 89:11302-11306; Stewart and Denell, 1993, Mol. Cell. Biol. 13:2524-2535).

Although examining homozygous mutant animals has allowed the successful identification of overproliferation mutations that cause late larval and pupal lethality, mutations that cause lethality at early developmental stages are unlikely to be recovered by this approach. The present invention solves this problem by providing a method for identifying tumor suppressor genes that does not exclude genes that when mutated cause lethality in early developmental stages, and provides genes thus identified with a fundamental role in regulation of cell proliferation.

The cessation of proliferative capacity by cells in culture is termed cellular senescence. Cellular senescence is used as an experimental model for cellular aging. Normal vertebrate cells in culture have a finite lifespan in that they undergo a characteristic maximum number of population doublings. The maximum number of population doublings that a cell can undergo inversely correlates with the age of the human donor. Cells from many human tumors are immortal cell lines when grown in tissue culture, *i.e.*, they exhibit infinite or continuous cell growth, suggesting that overcoming senescence is part of carcinogenesis. (For the foregoing see Hubbard and Ozer, 1995, "Senescence and immortalization of human cells," in Cell Growth and Apoptosis, A Practical Approach, Ch. 12, Studzinski, G.P. (ed.), Oxford University Press Inc., New York, NY, pp. 229-248; Hubbard-Smith et al., 1992, Mol. Cell. Biol. 12:2273-2281). A comparative study of preimmortalized and immortalized human fibroblasts transformed with a defective SV40 genome has led to the suggestion that a chromosomal region at and/or distal to 6q21 plays a role in

immortalization of cells (Hubbard-Smith et al., 1992, Mol. Cell. Biol. 12:2273-2281).

Citation of references hereinabove shall not be construed as an admission that such references are prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention relates to nucleotide sequences of *lats* genes (*Drosophila*, human, and mouse *lats* and lats homologs of other species), and amino acid sequences of their encoded proteins, as well as derivatives (*e.g.*, fragments) and analogs thereof. Nucleic acids hybridizable to or complementary to the foregoing nucleotide sequences are also provided. In a specific embodiment, the lats protein is a human protein.

The invention also relates to a method of identifying tumor suppressor genes that does not exclude from identification genes that cause lethality at early developmental stages, thus overcoming the limitations of prior art methods. The method thus allows the identification of genes that regulate cell proliferation and that act at early developmental stages. The genes which thus can be identified play a fundamental role in regulation of cell proliferation such that their dysfunction (*e.g.*, by lack of expression or mutation) leads to overproliferation and cancer.

Lats is a gene provided by the present invention, identified by the method of the invention, that acts to inhibit cell proliferation, and that plays a crucial role throughout development.

The invention also relates to lats derivatives and analogs of the invention which are functionally active, i.e., they are capable of displaying one or more known functional activities associated with a full-length (wild-type) lats protein. Such functional activities include but are not limited to kinase activity, antigenicity [ability to bind (or comp te with lats for binding) to an anti-lats antibody],

immunogenicity (ability to generate antibody which binds to lats), and ability to bind (or compet with lats for binding) to a receptor/ligand for lats (*e.g.*, a SH3 domain-containing protein).

The invention further relates to fragments (and derivatives and analogs thereof) of lats which comprise one or more domains of a lats protein.

Antibodies to lats, and lats derivatives and analogs, are additionally provided.

Methods of production of the lats proteins, derivatives and analogs, *e.g.*, by recombinant means, are also provided.

The present invention also relates to therapeutic and diagnostic methods and compositions based on lats proteins and nucleic acids. Therapeutic compounds of the invention include but are not limited to lats proteins and analogs and derivatives (including fragments) thereof; antibodies thereto; nucleic acids encoding the lats proteins, analogs, or derivatives; and *lats* antisense nucleic acids.

The invention provides for treatment of disorders of overproliferation (*e.g.*, cancer and hyperproliferative disorders) by administering compounds that promote lats activity (*e.g.*, lats, an agonist of lats; nucleic acids that encode lats).

The invention also provides methods of treatment of disorders involving deficient cell proliferation (growth) or in which cell proliferation is otherwise desired (*e.g.*, degenerative disorders, growth deficiencies, lesions, physical trauma) by administering compounds that antagonize, (inhibit) lats function (*e.g.*, antibodies, antisense nucleic acids).

In a specific embodiment, lats function is antagonized in order to inhibit cellular senescence, *in vivo* or *in vitro*.

Antagonizing lats function can also be done to grow larger animals and plants, *e.g.*, those used as food or mat rial sourc s.

Animal models, diagnostic methods and screening methods for predisposition to disorders, and methods to id ntify lats agonists and antagonists, are also provided by th inv ntion.

3.1. DEFINITIONS

As used herein, underscoring or italicizing the name of a gene shall indicate the gene, in contrast to its encoded protein product which is indicated by the name of the gene in the absence of any underscoring or italicizing. For example, "*lats*" shall mean the *lats* gene, whereas "lats" shall indicate the protein product of the *lats* gene.

4. DESCRIPTION OF THE FIGURES

Figure 1. Identifying overproliferation mutations in mosaic flies. (A) Although animals that are homozygous for a lethal mutation could die at an early developmental stage, mosaic flies carrying clones of cells that are homozygous for the same mutation could live. One can identify potential tumor suppressors by generating and examining clones of overproliferated mutant cells in mosaic animals. The genetic constitution of these mosaic flies is similar to the mosaicism of the tumor patients. (B) Genetic scheme. The P-element insertions carrying the FLP recombinase (hsFLP; Golic and Lindquist, 1989, Cell 59:499-509), its target site, FRT (solid arrows, Xu and Rubin, 1993, Development 117:1223-1237), the *yellow*$^+$ and *mini-white*$^+$ marker genes (y^+ and *mini*-w^+, open arrows) are indicated. Mutagenized males were crossed to females to produce heterozygous embryos. Clones of cells homozygous for the induced mutations were generated in developing first-instar larvae by mitotic recombination at the FRT sites induced with the FLP recombinase. Mosaic adults were examined for overproliferated mutant patches (w^-, y^-). Individuals carrying clones of interest were then mated to recover the mutations of interest in the next generation (Xu and Rubin,

1993, Developm nt 117:1223-1237; Xu and Harrison, 1994; Methods in Cell Biology 44:655-682). Clones of ommatidia derived from fast proliferating mutant cells were identified since th y w re larger than their darkly pigmented wt (wild-type) twin-spot clones (*mini-w*$^{+}$/*mini-w*$^{+}$).

Figure 2. Mutant phenotypes. (A) A clone of unpatterned, overproliferated *lats* mutant cells in the eye. (B) Induced at the same stage, the 93B mutant cells formed a less overproliferated clone. (C) A third instar *lats*$^{e26-1}$ larva (right) was much larger than a wt sibling (left; at 18°C). (D) Wing discs from the larva in (C) (wt, top; *lats*$^{e26-1}$, bottom). (E) Dissected central nervous systems (wt, top; *lats*$^{e26-1}$, bottom). (F) A SEM (scanning electron microscope) view of a *lats* clone near the eye. (G) A closer view of a region in (F) showing the irregularity of the sizes and shapes of the mutant cells. (H) A plastic section of a mutant clone similar to the one in (F). Cells seem to be "budding" out of the surface to form new proliferating lobes (arrows). (I) A *lats* clone on the back. The boxed area is shown in (J). The bristles in the mutant clone are short, bent and often split (arrows). (K) A closer view of the hairs in a *lats* clone on the body showing enlarged bases and bent tips. (L) A section of a *lats* clone on the back showing extra cuticle deposits (arrows). All the mutant clones were induced with *lats*^{x1} unless stated differently.

Figure 3. Organization of the *Drosophila lats* gene. The genomic restriction map of the *lats* region is aligned with the *lats* 5.7 kb transcript unit. The direction of transcription is indicated with large arrows. The sizes of the *lats* introns are as follows: intron 1 (5.0 kb), intron 2 (5.8 kb), intron 3 (68 bp), intron 4 (63 bp), intron 5 (64 bp), intron 6 (61 bp), intron 7 (62 bp). The genomic DNA from +7.5 (*Bgl*II) to -4.2 (*Eco*RI) was used to screen a total imaginal disc cDNA library, which isolated three groups of cDNAs: *lats*, T1, T2. The introns in the T2 transcript are not labeled. Only parts of the *zfh-1* (Fortini et al., 1991, Mechan. Dev. 34:113-122) and T1 transcripts are

indicated. The locations of the P-element insertion (*lats*^{P1}), th deletions in th five excision alleles (*lats*$^{e7-2, e78, e100, e119, e148}$) and in *lats*^{a1}, *lats*^{a4} are indicated at the bottom. The slash indicates a gap in the genomic map. Restriction sites: *Eco*RI (small open arrow), *Bgl*II (open box) and *Bam*HI (open circle). The *Bgl*II site at the -0.5 position of the CLT-52 clone is not present in other genomic DNA. A scale is labeled under the restriction map.

Figure 4. RNA blot analysis of the *Drosophila lats* mRNA. Five μg of poly(A)$^+$ RNA isolated from various developmental stages was separated on a 1% agarose gel, and hybridized with ^{32}P-labeled 5' end 1 kb probe from the *Drosophila lats* cDNA. E0-2 hrs, E2-4 hrs, E4-6 hrs, E6-8 hrs, E8-16 hrs and E16-24 hrs indicate the age of the embryos in hours. RNA from first, second and third instar larvae is denoted by L1, L2, and L3, respectively. The numbers and arrows on the right correspond to the size and location of the RNA standards. A 5.7 kb RNA was found in all the developmental stages, whereas a 4.7 kb RNA was predominantly present in 0 to 4 hour old embryos. The blot was also hybridized with DNA from the ribosomal protein gene, RNA1.

Figure 5. Composite cDNA sequence of the *Drosophila lats* gene. The entire cDNA sequence (SEQ ID NO:1) corresponding to the 5.7 kb *lats* RNA is shown. This nucleotide sequence is a composite of two cDNA clones (nucleotide 1-191 from cDNA 9 and the rest from cDNA A2). The sequence of the corresponding genomic DNA has been determined and is identical to the cDNA sequence except where indicated (above the cDNA sequence). The predicted amino acid sequence (SEQ ID NO:2) is shown below the cDNA sequence. The opa repeat is indicated by the heavy bar. The location of the putative SH3 binding site and the RERDQ peptides are designated by dashed lines. The two sites that match the polyadenylation signal consensus sequence are underlined. The second site is located at 12 bp away from the 3' end of the cDNA. The locations of the introns are indicat d by v rtical arrows. The underlined 141 bp sequ nce at the 3'

end of the *lats* transcript is identical to the 5' end untranslat d sequenc of the class I transcript of the *Drosophila* phospholipase C gene, *plc-21*. The location of the 446 bp d l tion in the *lats*^{a1} all le is also indicated.

Figure 6. Schematic of the *Drosophila* lats predicted protein (SEQ ID NO:2) and the related proteins (A) and sequence comparison of the proteins homologous to lats (B). In Fig. 6A, solid, hatched, open and shaded boxes denote putative SH3 binding site, opa repeat, RERDQ peptide and kinase domain in the lats protein, respectively. The Dbf20, Dbf2 and COT-1 proteins are illustrated at the bottom. The regions that are homologous to lats are indicated by shaded boxes. The degrees of sequence similarity (percentage of identical sequences inside parentheses; percentage of identical or conservative substitutions outside parentheses) between lats and the three related proteins are indicated above the corresponding regions of these proteins. In Fig. 6B, the carboxy-terminal half of lats is compared to the six most related proteins that are revealed by blastp (a software program that searches for protein sequence homologies) search as of Sept. 1, 1994. Neurospora cot-1 (SEQ ID NO:11); tobacco PKTL7 (SEQ ID NO:12); common ice plant protein kinase (SEQ ID NO:13); spinach protein kinase (SEQ ID NO:14); yeast Dbf-20 (SEQ ID NO:15); yeast Dbf2 (SEQ ID NO:16). Amino acid residues identical to lats are highlighted. Numbers at the beginning of every sequence refer to the position of that amino acid within the total protein sequence. The boundary of the kinase domain is defined according to Hanks et al. (1988, Science 241:42-52). The location of a region of about 40 amino acid residues that is not conserved among the proteins is indicated by the heavy bar above the sequence. The sequence of PKTL7 from tobacco, *Nicotiana tabacum*, was submitted to Genbank by Huang,Y. (X71057). Both the sequence of the protein kinase from spinach, *Spinacia oleracea*, and the sequence of the protein kinase from common ice plant, *Mesembryanthemum crystallinum*, were submitted to Genbank by

Baur, B., Winter, K., Fischer, K. and Dietz, K. (Z30329 and Z30330).

Figur 7. cDNA sequence (SEQ ID NO:5) and deduced prot in sequence (SEQ ID NO:6) of a mouse lats homolog, m-lats.

Figure 8. cDNA sequence (SEQ ID NO:7) and deduced protein sequence (SEQ ID NO:8) of a mouse lats homolog, m-lats2.

Figure 9. cDNA sequence (SEQ ID NO:3) and deduced protein sequence (SEQ ID NO:4) of a human lats homolog, h-lats.

Figure 10. Schematic diagram of plasmid pBS(KS)-h-lats, containing the full length coding sequence of the h-lats cDNA.

Figure 11. Alignment of the h-lats protein sequence (SEQ ID NO:4) (upper case letters) with the m-lats protein sequence (SEQ ID NO:6) (lower case letters). A dot indicates amino acid identity; a dash indicates a deletion relative to the sequence on the line above. The amino-terminal portion of the m-lats protein is not shown due to the missing 5′ end of the m-lats cDNA coding region.

Figure 12. Alignment of the h-lats protein sequence (SEQ ID NO:4) (upper case letters) with the m-lats2 protein sequence (SEQ ID NO:8) (lower case letters). A dot indicates amino acid identity; a dash indicates a deletion relative to the sequence on the line above. The amino-terminal portion of the m-lats2 protein is not shown due to the missing 5′ end of the m-lats2 cDNA coding region.

Figure 13. Alignment of the h-lats protein sequence (SEQ ID NO:4) (upper case letters) with the *Drosophila* lats protein sequence (SEQ ID NO:2) (lower case letters). A dot indicates amino acid identity; a dash indicates a deletion relative to the sequence on the line above. Insertions in the *Drosophila* sequence relative to the human sequence are indicated below the sequence line. Conserved domains are indicated. LSD2 = lats split domain 2; LSD2a = LSD2 anterior portion; LSD2p = LSD2 posterior

portion. The putative SH3-binding domain and th kinase domain ar shown. LSD1 = lats split domain 1; LSD1a = LSD1 ant rior p rtion; LSD1p = LSD1 posterior portion. LFD = lats flanking domain. LCD1 = lats C-t rminal domain 1; LCD2 = lats C-terminal domain 2; LCD3 = lats C-terminal domain 3.

Figure 14. Schematic diagram of plasmid pCaSpeR-hs-h-lats, an expression vector containing the full length coding sequence of the h-lats cDNA.

Figure 15. Northern blot analysis of h-lats expression in normal human tissues. A ^{32}P-labeled *Bam*HI fragment of h-lats was used as a probe for hybridization to polyA^{+} RNA from the normal human fetal and adult tissues indicated for each lane. The positions of standard molecular weight markers are shown at right. The positions of the h-lats RNA and of β-actin RNA (used as a standard) are shown.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to nucleotide sequences of *lats* genes, and amino acid sequences of their encoded proteins. The invention further relates to fragments and other derivatives, and analogs, of lats proteins. Nucleic acids encoding such fragments or derivatives are also within the scope of the invention. The invention provides *lats* genes and their encoded proteins of many different species. The *lats* genes of the invention include *Drosophila*, human, and mouse *lats* and related genes (homologs) in other species. In specific embodiments, the *lats* genes and proteins are from vertebrates, or more particularly, mammals. In a preferred embodiment of the invention, the *lats* genes and proteins are of human origin. Production of the foregoing proteins and derivatives, *e.g.*, by recombinant methods, is provided.

The invention also relates to a method of identifying tumor suppressor genes that does not exclude from identification genes that cause lethality at early developmental stages, thus overcoming the limitations of prior art methods. The method thus allows the identification

f genes that regulate cell proliferation and that act at early d v lopmental stages. The gen s which thus can be identified play a fundamental role in regulation of cell prolif ration such that their dysfunction (*e.g.*, due to lack of expression or mutation) leads to overproliferation and cancer.

Lats is a gene provided by the present invention, identified by the method of the invention, that acts to inhibit cell proliferation, and that plays a crucial role throughout development.

The invention also relates to lats derivatives and analogs of the invention which are functionally active, i.e., they are capable of displaying one or more known functional activities associated with a full-length (wild-type) lats protein. Such functional activities include but are not limited to kinase activity, antigenicity [ability to bind (or compete with lats for binding) to an anti-lats antibody], immunogenicity (ability to generate antibody which binds to lats), ability to bind (or compete with lats for binding) to an SH3-domain-containing protein or other ligand, ability to inhibit cell proliferation, tumor inhibition, etc.

The invention further relates to fragments (and derivatives and analogs thereof) of lats which comprise one or more domains of the lats protein.

Antibodies to lats, its derivatives and analogs, are additionally provided.

The present invention also relates to therapeutic and diagnostic methods and compositions based on lats proteins and nucleic acids and anti-lats antibodies. The invention provides for treatment of disorders of overproliferation (*e.g.*, cancer and hyperproliferative disorders) by administering compounds that promote lats activity (*e.g.*, lats proteins and functionally active analogs and derivatives (including fragments) thereof; nucleic acids encoding the lats proteins, analogs, or derivatives, agonists of lats).

The invention also provides methods of treatment of disord rs involving d ficient cell proliferation or in which cell proliferation (growth) is otherwise desirable (*e.g.*, growth deficiencies, degenerative disorders, lesions, physical trauma) by administering compounds that antagonize, or inhibit, lats function (*e.g.*, antibodies, lats antisense nucleic acids, lats derivatives that are dominant-negative protein kinases).

In a specific embodiment, lats function is antagonized in order to inhibit cellular senescence, *in vivo* or *in vitro*.

Inhibition of lats function can also be done to grow larger farm animals and plants.

Animal models, diagnostic methods and screening methods for predisposition to disorders are also provided by the invention.

The invention is illustrated by way of examples *infra* which disclose, *inter alia*, the cloning and characterization of *D. melanogaster lats* (Section 6); the cloning and characterization of mouse and human *lats* homologs (Section 7); the sequence and domain conservation among the *lats* homologs (Section 8); the functional interchangeability of the human and *Drosophila lats* homologs (Section 9); and the differentially decreased expression of human *lats* in human tumor cell lines (Section 10).

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections which follow.

5.1. ISOLATION OF THE *LATS* GENES

The invention relates to the nucleotide sequences of *lats* nucleic acids. In specific embodiments, *lats* nucleic acids comprise the cDNA sequences of SEQ ID NO:1, 3, 5, or 7, or the coding regions thereof, or nucleotide sequences acids encoding a lats protein (*e.g.*, a protein having the sequence of SEQ ID NO:2, 4, 6, or 8). The invention provides purified nucleic acids consisting of at least 8 nucleotides (*i.e.*, a

hybridizable portion) of a *lats* sequence; in other embodiments, the nucleic acids consist of at least 25 (continuous) nucleotides, 50 nucleotides, 100 nucleotides, 150 nucleotides, or 200 nucleotides of a *lats* sequence, or a full-length *lats* coding sequence. In another embodiment, the nucleic acids are smaller than 35, 200 or 500 nucleotides in length. Nucleic acids can be single or double stranded. The invention also relates to nucleic acids hybridizable to or complementary to the foregoing sequences. In specific aspects, nucleic acids are provided which comprise a sequence complementary to at least 10, 25, 50, 100, or 200 nucleotides or the entire coding region of a *lats* gene. In a specific embodiment, a nucleic acid which is hybridizable to a *lats* nucleic acid (*e.g.*, having sequence SEQ ID NO:3 or 7), or to a nucleic acid encoding a lats derivative, under conditions of low stringency is provided. By way of example and not limitation, procedures using such conditions of low stringency are as follows (see also Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. USA 78:6789-6792): Filters containing DNA are pretreated for 6 h at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 μg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 μg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 10^6 cpm ^{32}P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 40°C, and then washed for 1.5 h at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 h at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and reexposed to film. Other conditions of low stringency which may be used are well known in the art (*e.g.*, as employed for cross-species hybridizations).

In another sp cific embodiment, a nucleic acid which is hybridizabl to a *lats* nucleic acid under conditions of high stringency is provided. By way of example and not limitation, proc dur s using such conditions of high stringency are as follows: Prehybridization of filters containing DNA is carried out for 8 h to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 μg/ml denatured salmon sperm DNA. Filters are hybridized for 48 h at 65°C in prehybridization mixture containing 100 μg/ml denatured salmon sperm DNA and 5-20 X 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for 1 h in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1X SSC at 50°C for 45 min before autoradiography. Other conditions of high stringency which may be used are well known in the art.

In another specific embodiment, a nucleic acid, which is hybridizable to a *lats* nucleic acid under conditions of moderate stringency is provided (see, *e.g.*, Section 7.2).

Nucleic acids encoding derivatives and analogs of lats proteins (see Sections 5.6 and 5.6.1), and *lats* antisense nucleic acids (see Section 5.8.2.2.1) are additionally provided. As is readily apparent, as used herein, a "nucleic acid encoding a fragment or portion of a lats protein" shall be construed as referring to a nucleic acid encoding only the recited fragment or portion of the lats protein and not the other contiguous portions of the lats protein as a continuous sequence.

Fragments of *lats* nucleic acids comprising regions conserved between (with homology to) other *lats* nucleic acids, of the same or different species, are also provided. Nucleic acids encoding one or more lats domains are provided.

Specific embodiments for the cloning of a *lats* gene, presented as a particular example but not by way of limitation, follows:

For expression cloning (a technique commonly known in the art), an expression library is constructed by methods

known in the art. For example, mRNA (*e.g.*, human) is isolated, cDNA is made and ligated into an expression vector (*e.g.*, a bacteriophag derivative) such that it is capable of being expressed by the host cell into which it is then introduced. Various screening assays can then be used to select for the expressed lats product. In one embodiment, anti-lats antibodies can be used for selection.

In another embodiment, polymerase chain reaction (PCR) is used to amplify the desired sequence in a genomic or cDNA library, prior to selection. Oligonucleotide primers representing known *lats* sequences can be used as primers in PCR. In a preferred aspect, the oligonucleotide primers represent at least part of the lats conserved segments of strong homology between *lats* of different species (*e.g.*, LCD1, LCD2, kinase domain, LFD, SH3 binding domain, LSD1, and LSD2 domains; see, *e.g.*, Section 8 *infra*.) The synthetic oligonucleotides may be utilized as primers to amplify by PCR sequences from a source (RNA or DNA), preferably a cDNA library, of potential interest. PCR can be carried out, *e.g.*, by use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp™). The DNA being amplified can include mRNA or cDNA or genomic DNA from any eukaryotic species. One can choose to synthesize several different degenerate primers, for use in the PCR reactions. It is also possible to vary the stringency of hybridization conditions used in priming the PCR reactions, to allow for greater or lesser degrees of nucleotide sequence similarity between the known *lats* nucleotide sequence and the nucleic acid homolog being isolated. For cross species hybridization, low stringency conditions are preferred. For same species hybridization, moderately stringent conditions are preferred. After successful amplification of a segment of a *lats* homolog, that segment may be molecularly cloned and sequenced, and utilized as a probe to isolate a complete cDNA or genomic clone. This, in turn, will permit the determination of the gene's complete nucleotide sequence, the analysis of its expression, and the production of its protein product for functional

analysis, as described *infra*. In this fashion, additional gen s encoding lats proteins and lats analogs may be id ntified.

The above-methods are not meant to limit the following general description of methods by which clones of *lats* may be obtained.

Any eukaryotic cell potentially can serve as the nucleic acid source for the molecular cloning of the *lats* gene. The nucleic acid sequences encoding lats can be isolated from vertebrate, mammalian, human, porcine, bovine, feline, avian, equine, canine, as well as additional primate sources, insects, plants, etc. The DNA may be obtained by standard procedures known in the art from cloned DNA (*e.g.*, a DNA "library"), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell. (See, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II.) Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will contain only exon sequences. Whatever the source, the gene should be molecularly cloned into a suitable vector for propagation of the gene.

In the molecular cloning of the gene from genomic DNA, DNA fragments are generated, some of which will encode the desired gene. The DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNAse in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis and column chromatography.

Once the DNA fragments are generated, identification of th sp cific DNA fragment containing the

d sir d gene may b accomplished in a number of ways. For example, if an amount of a portion of a *lats* (of any species) gen or its specific RNA, or a fragment thereof (see Section 5.6), is available and can be purified and labeled, the generated DNA fragments may be screened by nucleic acid hybridization to the labeled probe (Benton, W. and Davis, R., 1977, Science 196:180; Grunstein, M. And Hogness, D., 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961). Such a procedure is presented by way of example in Section 7 *infra*. Those DNA fragments with substantial homology to the probe will hybridize. It is also possible to identify the appropriate fragment by restriction enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map if such is available. Further selection can be carried out on the basis of the properties of the gene. Alternatively, the presence of the gene may be detected by assays based on the physical, chemical, or immunological properties of its expressed product. For example, cDNA clones, or DNA clones which hybrid-select the proper mRNAs, can be selected which produce a protein that, *e.g.*, has similar or identical electrophoretic migration, isolectric focusing behavior, proteolytic digestion maps, kinase activity, inhibition of cell proliferation activity, substrate binding activity, or antigenic properties as known for lats. If an antibody to lats is available, the lats protein may be identified by binding of labeled antibody to the putatively lats synthesizing clones, in an ELISA (enzyme-linked immunosorbent assay)-type procedure.

The *lats* gene can also be identified by mRNA selection by nucleic acid hybridization followed by *in vitro* translation. In this procedure, fragments are used to isolate complementary mRNAs by hybridization. Such DNA fragments may represent available, purified *lats* DNA of another species (*e.g.*, *Drosophila*, mouse, human). Immunoprecipitation analysis or functional assays (*e.g.*, aggregation ability *in vitro*; binding to receptor; se *infra*) of th *in vitro* translation products of the isolated products

of the isolated mRNAs identifies the mRNA and, therefore, the compl mentary DNA fragments that contain the desired s qu nces. In addition, specific mRNAs may be selected by adsorption of polysomes isolated from cells to immobilized antibodies specifically directed against lats protein. A radiolabelled *lats* cDNA can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabelled mRNA or cDNA may then be used as a probe to identify the *lats* DNA fragments from among other genomic DNA fragments.

Alternatives to isolating the *lats* genomic DNA include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA which encodes the lats protein. For example, RNA for cDNA cloning of the *lats* gene can be isolated from cells which express *lats*. Other methods are possible and within the scope of the invention.

The identified and isolated gene can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as PBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene). The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequ nces. In an alternative method, the cleaved vector and *lats* gene may be modified by

homopolym ric tailing. Recombinant molecules can be introduc d into host cells via transformation, transfection, inf ction, el ctroporation, etc., so that many copies of the g ne sequence are generated.

In an alternative method, the desired gene may be identified and isolated after insertion into a suitable cloning vector in a "shot gun" approach. Enrichment for the desired gene, for example, by size fractionization, can be done before insertion into the cloning vector.

In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate the isolated *lats* gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

The lats sequences provided by the instant invention include those nucleotide sequences encoding substantially the same amino acid sequences as found in native lats proteins, and those encoded amino acid sequences with functionally equivalent amino acids, as well as those encoding other lats derivatives or analogs, as described in Sections 5.6 and 5.6.1 *infra* for lats derivatives and analogs.

5.2. EXPRESSION OF THE *LATS* GENES

The nucleotide sequence coding for a lats protein or a functionally active analog or fragment or other derivative thereof (see Section 5.6), can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can also be supplied by the native *lats* gene and/or its flanking regions. A variety of host-v ctor systems may be utilized to express the protein-coding sequence. These include but are not

limited to mammalian cell systems inf cted with virus (*e.g.*, vaccinia virus, ad novirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as y ast containing yeast v ctors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used. In specific embodiments, the human *lats* gene is expressed, or a sequence encoding a functionally active portion of human lats. In yet another embodiment, a fragment of lats comprising a domain of the lats protein is expressed.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of nucleic acid sequence encoding a lats protein or peptide fragment may be regulated by a second nucleic acid sequence so that the lats protein or peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of a lats protein may be controlled by any promoter/enhancer element known in the art. In a specific embodiment, the promoter is not a native *lats* gene promoter. Promoters which may be used to control *lats* expression include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as th β-lactamase promoter (Villa-Kamaroff, et al.,

1978, Pr c. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the *tac* promoter (D Bo r, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner, et al., 1981, Nucl. Acids Res. 9:2871), and the promoter of th photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mous mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region

which is active in oligodendrocyte c lls in th brain (R adh ad et al., 1987, Cell 48:703-712); myosin light chain-2 gen contr l region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

In a specific embodiment, a vector is used that comprises a promoter operably linked to a lats-encoding nucleic acid, one or more origins of replication, and, optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene).

In a specific embodiment, an expression construct is made by subcloning a *lats* coding sequence into the *Eco*RI restriction site of each of the three pGEX vectors (Glutathione S-Transferase expression vectors; Smith and Johnson, 1988, Gene 7:31-40). This allows for the expression of the lats protein product from the subclone in the correct reading frame.

Expression vectors containing *lats* gene inserts can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a *lats* gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted *lats* gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of a *lats* gene in the vector. For example, if the *lats* gene is inserted within the marker gene sequence of the vector, recombinants containing the *lats* insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the lats product expressed by the

r combinant. Such assays can be based, for example, on the physical or functional properties of the lats protein in *in vitro* assay syst ms, *e.g.*, kinas activity, binding with anti-lats antibody, inhibition of cell proliferation.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered lats protein may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, glycosylation, phosphorylation of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian cells can be used to ensure "native" glycosylation of a heterologous protein. Furthermore, different vector/host expression systems may effect processing reactions to different extents.

In other sp cific embodiments, the lats protein, fragment, analog, or derivative may be expr ssed as a fusion,

r chimeric protein product (comprising the protein, fragm nt, analog, or derivative joined via a peptide bond to a h t rologous protein sequence (of a different protein)). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer.

Both cDNA and genomic sequences can be cloned and expressed.

5.3. IDENTIFICATION AND PURIFICATION OF THE *LATS* GENE PRODUCTS

In particular aspects, the invention provides amino acid sequences of lats, preferably human lats, and fragments and derivatives thereof which comprise an antigenic determinant (*i.e.*, can be recognized by an antibody) or which are otherwise functionally active, as well as nucleic acid sequences encoding the foregoing. "Functionally active" lats material as used herein refers to that material displaying one or more known functional activities associated with a full-length (wild-type) lats protein, *e.g.*, kinase activity, inhibition of cell proliferation, tumor inhibition, binding to an SH3-domain, binding to a lats substrate or lats binding partner, antigenicity (binding to an anti-lats antibody), immunogenicity, etc.

In specific embodiments, the invention provides fragments of a lats protein consisting of at least 6 amino acids, 10 amino acids, 50 amino acids, or of at least 75 amino acids. In other embodiments, the proteins comprise or consist essentially of a lats carboxy (C)-terminal domain 3 (LCD3), lats C-terminal domain 2 (LCD2), lats C-terminal domain 1 (LCD1), kinase domain, kinase subdomains, lats flanking domain (amino-terminal to the kinase domain), lats split domain 1 (LSD1), lats split domain 2 (LSD2),

SH3-binding domain, and opa r peat domain (s e Section 8 *infra*), or any combination of the foregoing, of a lats protein. Fragments, or proteins comprising fragments, lacking some or all of the foregoing regions of a lats protein are also provided. Nucleic acids encoding the foregoing are provided.

Once a recombinant which expresses the *lats* gene sequence is identified, the gene product can be analyzed. This is achieved by assays based on the physical or functional properties of the product, including radioactive labelling of the product followed by analysis by gel electrophoresis, immunoassay, etc.

Once the lats protein is identified, it may be isolated and purified by standard methods including chromatography (*e.g.*, ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. The functional properties may be evaluated using any suitable assay (see Section 5.7).

Alternatively, once a lats protein produced by a recombinant is identified, the amino acid sequence of the protein can be deduced from the nucleotide sequence of the chimeric gene contained in the recombinant. As a result, the protein can be synthesized by standard chemical methods known in the art (*e.g.*, see Hunkapiller, M., et al., 1984, Nature 310:105-111).

In another alternate embodiment, native lats proteins can be purified from natural sources, by standard methods such as those described above (*e.g.*, immunoaffinity purification).

In a specific embodiment of the present invention, such lats proteins, whether produced by recombinant DNA techniques or by chemical synthetic methods or by purification of native proteins, include but are not limited to those containing, as a primary amino acid sequence, all or part of the amino acid s qu nce substantially as depicted in Figure 9 (SEQ ID NO:4), as well as fragm nts and other

derivativ s, and analogs thereof, including proteins homologous thereto.

5.4. STRUCTURE OF THE *LATS* GENE AND PROTEIN

The structure of the *lats* gene and protein can be analyzed by various methods known in the art.

5.4.1. GENETIC ANALYSIS

The cloned DNA or cDNA corresponding to the *lats* gene can be analyzed by methods including but not limited to Southern hybridization (Southern, E.M., 1975, J. Mol. Biol. 98:503-517), Northern hybridization (see *e.g.*, Freeman et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:4094-4098), restriction endonuclease mapping (Maniatis, T., 1982, Molecular Cloning, A Laboratory, Cold Spring Harbor, New York), and DNA sequence analysis. Polymerase chain reaction (PCR; U.S. Patent Nos. 4,683,202, 4,683,195 and 4,889,818; Gyllenstein et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7652-7656; Ochman et al., 1988, Genetics 120:621-623; Loh et al., 1989, Science 243:217-220) followed by Southern hybridization with a *lats*-specific probe can allow the detection of the *lats* gene in DNA from various cell types. Methods of amplification other than PCR are commonly known and can also be employed. In one embodiment, Southern hybridization can be used to determine the genetic linkage of *lats*. Northern hybridization analysis can be used to determine the expression of the *lats* gene. Various cell types, at various states of development or activity can be tested for *lats* expression. The stringency of the hybridization conditions for both Southern and Northern hybridization can be manipulated to ensure detection of nucleic acids with the desired degree of relatedness to the specific *lats* probe used. Modifications of these methods and other methods commonly known in the art can be used.

Restriction endonuclease mapping can be used to roughly determine the genetic structure of the *lats* gene.

Restriction maps derived by restriction endonuclease cleavage can be confirm d by DNA sequence analysis.

DNA sequence analysis can b performed by any techniques known in the art, including but not limited to the method of Maxam and Gilbert (1980, Meth. Enzymol. 65:499-560), the Sanger dideoxy method (Sanger, F., et al., 1977, Proc. Natl. Acad. Sci. U.S.A. 74:5463), the use of T7 DNA polymerase (Tabor and Richardson, U.S. Patent No. 4,795,699), or use of an automated DNA sequenator (*e.g.*, Applied Biosystems, Foster City, CA).

5.4.2. PROTEIN ANALYSIS

The amino acid sequence of the lats protein can be derived by deduction from the DNA sequence, or alternatively, by direct sequencing of the protein, *e.g.*, with an automated amino acid sequencer.

The lats protein sequence can be further characterized by a hydrophilicity analysis (Hopp, T. and Woods, K., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:3824). A hydrophilicity profile can be used to identify the hydrophobic and hydrophilic regions of the lats protein and the corresponding regions of the gene sequence which encode such regions.

Secondary, structural analysis (Chou, P. and Fasman, G., 1974, Biochemistry 13:222) can also be done, to identify regions of lats that assume specific secondary structures.

Manipulation, translation, and secondary structure prediction, open reading frame prediction and plotting, as well as determination of sequence homologies, can also be accomplished using computer software programs available in the art.

Other methods of structural analysis can also be employed. These include but are not limited to X-ray crystallography (Engstom, A., 1974, Biochem. Exp. Biol. 11:7-13) and computer modeling (Fletterick, R. and Zoller, M. (ds.), 1986, Computer Graphics and Molecular Modeling, *in*

Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

5.5. GENERATION OF ANTIBODIES TO LATS PROTEINS AND DERIVATIVES THEREOF

According to the invention, lats protein, its fragments or other derivatives, or analogs thereof, may be used as an immunogen to generate antibodies which immunospecifically bind such an immunogen. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. In a specific embodiment, antibodies to a human lats protein are produced. In another embodiment, antibodies to a domain (*e.g.*, the SH3-binding domain) of a lats protein are produced. In a specific embodiment, fragments of a lats protein identified as hydrophilic are used as immunogens for antibody production.

Various procedures known in the art may be used for the production of polyclonal antibodies to a lats protein or derivative or analog. In a particular embodiment, rabbit polyclonal antibodies to an epitope of a lats protein encoded by a sequence of SEQ ID NOS:2, 4, 6 or 8, or a subsequence thereof, can be obtained. For the production of antibody, various host animals can be immunized by injection with the native lats protein, or a synthetic version, or derivative (*e.g.*, fragment) thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodi s directed toward a lats protein sequence or analog thereof, any

t chniqu which provides for the production of antibody m l cules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule specific for lats together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce lats-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for lats proteins, derivatives, or analogs.

Antibody fragments which contain the idiotype of the m lecule can be generated by known techniques. For xample, such fragments include but are not limited to: the

$F(ab')_2$ fragm nt which can be produced by pepsin digestion of th antibody mol cul ; the Fab' fragments which can be generated by r ducing the disulfide bridges of th $F(ab')_2$ fragment, the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.* ELISA (enzyme-linked immunosorbent assay). For example, to select antibodies which recognize a specific domain of a lats protein, one may assay generated hybridomas for a product which binds to a lats fragment containing such domain. For selection of an antibody that specifically binds a first lats homolog but which does not specifically bind a different lats homolog, one can select on the basis of positive binding to the first lats homolog and a lack of binding to the second lats homolog.

Antibodies specific to a domain of a lats protein are also provided.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the lats protein sequences of the invention, *e.g.*, for imaging these proteins, measuring levels thereof in appropriate physiological samples, in diagnostic methods, etc.

In another embodiment of the invention (see *infra*), anti-lats antibodies and fragments thereof containing the binding domain are Therapeutics.

5.6. LATS PROTEINS, DERIVATIVES AND ANALOGS

The invention further relates to lats proteins, and derivatives (including but not limited to fragments) and analogs of lats proteins. Nucleic acids encoding lats protein derivatives and protein analogs are also provided. In one embodiment, the lats proteins are encoded by the lats nucleic acids described in Section 5.1 *supra*. In particular aspects, the proteins, derivatives, or analogs are of lats

pr teins of animals, *.g.*, fly, frog, mouse, rat, pig, cow, dog, m nkey, human, or of plants.

The production and us f derivatives and analogs related to *lats* are within the scope of the present invention. In a specific embodiment, the derivative or analog is functionally active, i.e., capable of exhibiting one or more functional activities associated with a full-length, wild-type lats protein. As one example, such derivatives or analogs which have the desired immunogenicity or antigenicity can be used, for example, in immunoassays, for immunization, for inhibition of lats activity, etc. As another example, such derivatives or analogs which have the desired kinase activity, or which are phosphorylated or dephosphorylated, are provided. Derivatives or analogs that retain, or alternatively lack or inhibit, a desired lats property of interest (*e.g.*, binding to an SH3-domain-containing protein or other lats binding partner, kinase activity, inhibition of cell proliferation, tumor inhibition), can be used as inducers, or inhibitors, respectively, of such property and its physiological correlates. A specific embodiment relates to a lats fragment that can be bound by an anti-lats antibody. Derivatives or analogs of lats can be tested for the desired activity by procedures known in the art, including but not limited to the assays described in Sections 5.7 and 5.9.

In particular, lats derivatives can be made by altering *lats* sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as a *lats* gene may be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of *lats* genes which are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the lats derivatives of th invention include, but

are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a lats protein including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

In a specific embodiment of the invention, proteins consisting of or comprising a fragment of a lats protein consisting of at least 10 (continuous) amino acids of the lats protein is provided. In other embodiments, the fragment consists of at least 20 or 50 amino acids of the lats protein. In specific embodiments, such fragments are not larger than 35, 100 or 200 amino acids. Derivatives or analogs of lats include but are not limited to those molecules comprising regions that are substantially homologous to lats or fragments thereof (*e.g.*, in various embodiments, at least 60% or 70% or 80% or 90% or 95% identity over an amino acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art) or whose encoding nucleic acid is capable of hybridizing to a coding *lats* sequence, under stringent, moderately stringent, or nonstringent conditions.

Th lats d rivatives and analogs of the inv ntion can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned *lats* gene sequence can be modified by any of numerous strategies known in the art (Maniatis, T., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of the gene encoding a derivative or analog of lats, care should be taken to ensure that the modified gene remains within the same translational reading frame as lats, uninterrupted by translational stop signals, in the gene region where the desired lats activity is encoded.

Additionally, the lats-encoding nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem 253:6551), use of TAB® linkers (Pharmacia), etc.

Manipulations of the lats sequence may also be made at the protein level. Included within the scope of the invention are lats protein fragments or other derivatives or analogs which are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous ch mical modifications may be carried out by known techniques, including but not limited to specific chemical

cleavag by cyanog n bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_4$; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc.

In addition, analogs and derivatives of lats can be chemically synthesized. For example, a peptide corresponding to a portion of a lats protein which comprises the desired domain (see Section 5.6.1), or which mediates the desired activity *in vitro*, can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the lats sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ-Abu, ε-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

In a specific embodiment, the lats derivative is a chimeric, or fusion, protein comprising a lats protein or fragment thereof (preferably consisting of at least a domain or motif of the lats protein, or at least 10 amino acids of the lats protein) joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different protein. In one embodiment, such a chimeric protein is produced by recombinant expression of a nucleic acid encoding the protein (comprising a lats-coding sequence joined in-frame to a coding sequence for a different protein). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the

proper coding frame, and expressing the chimeric product by m thods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer. Chimeric genes comprising portions of *lats* fused to any heterologous protein-encoding sequences may be constructed. A specific embodiment relates to a chimeric protein comprising a fragment of lats of at least six amino acids.

In another specific embodiment, the lats derivative is a molecule comprising a region of homology with a lats protein. By way of example, in various embodiments, a first protein region can be considered "homologous" to a second protein region when the amino acid sequence of the first region is at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, or 95% identical, when compared to any sequence in the second region of an equal number of amino acids as the number contained in the first region or when compared to an aligned sequence of the second region that has been aligned by a computer homology program known in the art. For example, a molecule can comprise one or more regions homologous to a lats domain (see Section 5.6.1) or a portion thereof.

Other specific embodiments of derivatives and analogs are described in the subsection below and examples sections *infra*.

5.6.1. DERIVATIVES OF LATS CONTAINING ONE OR MORE DOMAINS OF THE PROTEIN

In a specific embodiment, the invention relates to lats derivatives and analogs, in particular lats fragments and derivatives of such fragments, that comprise, or alternatively consist of, one or more domains of a lats protein, including but not limited to a lats C-terminal domain 3 (LCD3), lats C-terminal domain 2 (LCD2), lats C-terminal domain 1 (LCD1), kinase domain, kinase subdomains, lats flanking domain (LFD) (amino-terminal to the kinase domain), lats split domain 1 (LSD1), lats split domain 2 (LSD2), SH3-binding domain, and opa repeat domain, functional

(*e.g.*, binding) fragments of any of the foregoing, or any combination of the foregoing. In particular examples relating to the human, mouse and *Drosophila* lats proteins, such domains are identifi d in Examples Sections 6 and 8, and in Figures 6A, 6B, and 13.

A specific embodiment relates to molecules comprising specific fragments of lats that are those fragments in the respective lats protein most homologous to specific fragments of a human or mouse lats protein. A fragment comprising a domain of a lats homolog can be identified by protein analysis methods as described in Sections 5.3.2 or 6.

In a specific embodiment, a lats protein, derivative or analog is provided that has a kinase domain and has a phosphorylated serine situated within 20 residues upstream of an Ala-Pro-Glu consensus in subdomain eight of its kinase domain. In another embodiment, a lats protein derivative or analog is provided with a kinase domain and with a dephosphorylated serine situated within 20 residues upstream of an Ala-Pro-Glu consensus in subdomain eight of its kinase domain, or in which the serine situated within 20 residues upstream of that consensus has been deleted or substituted by another amino acid. In a specific embodiment, the invention provides various phosphorylated and dephosphorylated forms of the lats protein, derivative, or analog that are active kinase forms. Both phosphorylation and dephosphorylation of lats at different residues could potentially activate lats. In another specific embodiment, the invention provides various phosphorylated and dephosphorylated forms of the lats protein, derivative or analog that are inactive kinase forms. Phosphorylation can be carried out by any methods known in the art, *e.g.*, by use of a kinase. Dephosphorylation can be carried out by use of any methods known in the art, *e.g.*, by use of a phosphatase.

Another specific embodiment relates to a derivative or analog of a lats protein that is a dominant-active protein kinase. Such a derivative or analog comprises a lats kinase

domain that has been mutated so as to be dominantly active (xhibit constitutiv ly active kinase activity). It is known that acidic residues such as Glu and Asp sometimes mimic a phosphorylated residue, and changing the phosphorylatable Ser or Thr residue in subdomain eight into a Glu or Asp residue has been previously used to produce constitutively active kinases (Mansour et al., 1994, Science 265:966-970). Thus, changing a serine or threonine residue situated within 20 residues upstream of an Ala-Pro-Glu consensus in subdomain eight of a lats kinase domain into another residue (*e.g.*, Glu, Asp) may be used to make a dominant-active lats protein kinase. For example, changing Ser914 in *Drosophila* lats, or changing Ser909 in h-lats, into a Glu residue could produce a dominant active lats kinase.

Another specific embodiment relates to a derivative or analog of lats that is a dominant-negative protein kinase. Protein kinases can be mutated into dominant negative forms. Expression of a dominant negative protein kinase can suppress the activity of the wild-type form of the same kinase. Dominant negative forms of protein kinases are often obtained by expressing an inactive form of a kinase (Milarski and Saltiel, 1994, J. Biol. Chem. 269(33):21239-21243) or by expressing a noncatalytic domain of a kinase (Lu and Means, 1994, EMBO J. 12:2103-2113; Yarden et al., 1992, EMBO J. 11:2159-2166). Thus, a lats dominant-negative kinase can be obtained by mutating the kinase domain so as to be inactive (*e.g.*, by deletion and/or point mutation). By way of example, a lats derivative that is a dominant-negative kinase is a lats protein that lacks a kinase domain but comprises one or more of the other domains of the lats protein; *e.g.*, a lats protein derivative truncated at about the beginning of the kinase domain (*i.e.*, a lats fragment containing only sequences amino-terminal to the kinase domain). By way of another example, a lats derivative that is a dominant-negative kinase is a lats protein in which one of the residues conserved among s rine/threonine kinases (see Hanks

et al., 1988, Science 241:42-52) is mutated (del ted or substituted by a different residue).

In another specific embodiment, a molecule is provided that comprises one or more domains (or functional portion thereof) of a lats protein but that also lacks one or more domains (or functional portion thereof) of a lats protein. In particular examples, lats protein derivatives are provided that lack an opa repeat domain. By way of another example, such a protein may also lack all or a portion of the kinase domain, but retain at least the SH3-binding domain of a lats protein. In another embodiment, a molecule is provided that comprises one or more domains (or functional portion thereof) of a lats protein, and that has one or more mutant (*e.g.*, due to deletion or point mutation(s)) domains of a lats protein (*e.g.*, such that the mutant domain has decreased function). By way of example, the kinase domain may be mutant so as to have reduced, absent, or increased kinase activity.

5.7. ASSAYS OF LATS PROTEINS, DERIVATIVES AND ANALOGS

The functional activity of lats proteins, derivatives and analogs can be assayed by various methods.

For example, in one embodiment, where one is assaying for the ability to bind or compete with wild-type lats for binding to anti-lats antibody, various immunoassays known in the art can be used, including but not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In

on mbodim nt, antibody binding is detected by detecting a lab l n the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labelled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

In another embodiment, where a lats-binding protein is identified, the binding can be assayed, *e.g.*, by means well-known in the art. In another embodiment, physiological correlates of lats binding to its substrates (signal transduction) can be assayed.

In another embodiment, kinase assays can be used to measure lats kinase activity. Such assays can be carried out by methods well known in the art. By way of example, a lats protein is contacted with a substrate (*e.g.*, a known substrate of serine/threonine kinases) in the presence of a ^{32}P-labeled phosphate donor, and any phosphorylation of the substrate is detected or measured.

In another embodiment, in insect or other model systems, genetic studies can be done to study the phenotypic effect of a lats mutant that is a derivative or analog of wild-type *lats* (see Section 6, *infra*).

In addition, assays that can be used to detect or measure the ability to inhibit, or alternatively promote, cell proliferation are described in Section 5.9.

Other methods will be known to the skilled artisan and are within the scope of the invention.

5.8. THERAPEUTIC USES

The invention provides for treatment or prevention of various diseases and disorders by administration of a therapeutic compound (termed herein "Therapeutic"). Such "Therapeutics" include but are not limited to: lats proteins and analogs and derivatives (including fragments) thereof (*e.g.*, as described her inabove); antibodies thereto (as

described hereinabove); nucleic acids encoding the lats prot ins, analogs, or derivatives (.*g*., as d scribed h r inabove); *lats* antisense nucleic acids, and lats agonists and antagonists. Disorders involving c ll overproliferation are treated or prevented by administration of a Therapeutic that promotes lats function. Disorders in which cell proliferation is deficient or is desired are treated or prevented by administration of a Therapeutic that antagonizes (inhibits) lats function. The above is described in detail in the subsections below.

Generally, administration of products of a species origin or species reactivity (in the case of antibodies) that is the same species as that of the patient is preferred. Thus, in a preferred embodiment, a human lats protein, derivative, or analog, or nucleic acid, or an antibody to a human lats protein, is therapeutically or prophylactically administered to a human patient.

Additional descriptions and sources of Therapeutics that can be used according to the invention are found in Sections 5.1 through 5.7 herein.

5.8.1. TREATMENT AND PREVENTION OF DISORDERS INVOLVING OVERPROLIFERATION OF CELLS

Diseases and disorders involving cell overproliferation are treated or prevented by administration of a Therapeutic that promotes (*i.e.*, increases or supplies) lats function. Examples of such a Therapeutic include but are not limited to lats proteins, derivatives, or fragments that are functionally active, particularly that are active in inhibiting cell proliferation (*e.g.*, as demonstrated in *in vitro* assays or in animal models or in *Drosophila*), and nucleic acids encoding a lats protein or functionally active derivative or fragment thereof (*e.g.*, for use in gene therapy). Other Therapeutics that can be used, *e.g.*, lats agonists, can be identified using *in vitro* assays or animal models, or assays in *Drosophila*, examples of which are described *infra*.

In sp cific embodiments, Therapeutics that promote lats function ar administered therapeutically (including prophylactically): (1) in diseases or disorders involving an abs nce or decreased (relative to n rmal or desired) level of lats protein or function, for example, in patients where lats protein is lacking, genetically defective, biologically inactive or underactive, or underexpressed; or (2) in diseases or disorders wherein *in vitro* (or *in vivo*) assays (see *infra*) indicate the utility of lats agonist administration. The absence or decreased level in lats protein or function can be readily detected, *e.g.*, by obtaining a patient tissue sample (*e.g.*, from biopsy tissue) and assaying it *in vitro* for RNA or protein levels, structure and/or activity of the expressed lats RNA or protein. Many methods standard in the art can be thus employed, including but not limited to kinase assays, immunoassays to detect and/or visualize lats protein (*e.g.*, Western blot, immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunocytochemistry, etc.) and/or hybridization assays to detect lats expression by detecting and/or visualizing lats mRNA (*e.g.*, Northern assays, dot blots, *in situ* hybridization, etc.), etc.

Diseases and disorders involving cell overproliferation that can be treated or prevented include but are not limited to malignancies, premalignant conditions (*e.g.*, hyperplasia, metaplasia, dysplasia), benign tumors, hyperproliferative disorders, benign dysproliferative disorders, etc. Examples of these are detailed below.

In a specific embodiment, the Therapeutic used, that promotes lats function, is a lats protein, derivative or analog comprising a lats kinase domain (and optionally also a lats LFD, or the remainder of the lats sequence) in which a serine within 20 residues upstream of the Ala-Pro-Glu consensus in subdomain eight of the kinase domain is phosphorylated or substituted by another residue (*e.g.*, Glu, Asp).

In another specific emb diment, the Therapeutic used, that promotes lats function, is a derivative or analog comprising a kinase domain of a lats protein that has been mutated so as to be dominantly active.

5.8.1.1. MALIGNANCIES

Malignancies and related disorders that can be treated or prevented by administration of a Therapeutic that promotes lats function include but are not limited to those listed in Table 1 (for a review of such disorders, see Fishman et al., 1985, *Medicine*, 2d Ed., J.B. Lippincott Co., Philadelphia):

TABLE 1
MALIGNANCIES AND RELATED DISORDERS

- Leukemia
  - acute leukemia
    - acute lymphocytic leukemia
    - acute myelocytic leukemia
      - myeloblastic
      - promyelocytic
      - myelomonocytic
      - monocytic
      - erythroleukemia
  - chronic leukemia
    - chronic myelocytic (granulocytic) leukemia
    - chronic lymphocytic leukemia
- Polycythemia vera
- Lymphoma
  - Hodgkin's disease
  - non-Hodgkin's disease
- Multiple myeloma
- Waldenström's macroglobulinemia
- Heavy chain disease
- Solid tumors
  - sarcomas and carcinomas
    - fibrosarcoma
    - myxosarcoma
    - liposarcoma
    - chondrosarcoma
    - osteogenic sarcoma
    - chordoma
    - angiosarcoma
    - endotheliosarcoma
    - lymphangiosarcoma

lymphangioendoth liosarcoma
synovioma
mesothelioma
Ewing's tumor
l iomyosarcoma
rhabdomyosarcoma
colon carcinoma
pancreatic cancer
breast cancer
ovarian cancer
prostate cancer
squamous cell carcinoma
basal cell carcinoma
adenocarcinoma
sweat gland carcinoma
sebaceous gland carcinoma
papillary carcinoma
papillary adenocarcinomas
cystadenocarcinoma
medullary carcinoma
bronchogenic carcinoma
renal cell carcinoma
hepatoma
bile duct carcinoma
choriocarcinoma
seminoma
embryonal carcinoma
Wilms' tumor
cervical cancer
uterine cancer
testicular tumor
lung carcinoma
small cell lung carcinoma
bladder carcinoma
epithelial carcinoma
glioma
astrocytoma
medulloblastoma
craniopharyngioma
ependymoma
pinealoma
hemangioblastoma
acoustic neuroma
oligodendroglioma
menangioma
melanoma
neuroblastoma
retinoblastoma

In specific embodiments, malignancy or dysproliferative changes (such as metaplasias and

dysplasias), or hyperproliferative disorders, are treated or pr vented in the bladder, br ast, colon, lung, melanoma, pancreas, or uterus. In other specific embodiments, sarcoma, or l uk mia is treated or prevented.

5.8.1.2. PREMALIGNANT CONDITIONS

The Therapeutics of the invention that promote lats activity can also be administered to treat premalignant conditions and to prevent progression to a neoplastic or malignant state, including but not limited to those disorders listed in Table 1. Such prophylactic or therapeutic use is indicated in conditions known or suspected of preceding progression to neoplasia or cancer, in particular, where non-neoplastic cell growth consisting of hyperplasia, metaplasia, or most particularly, dysplasia has occurred (for review of such abnormal growth conditions, see Robbins and Angell, 1976, *Basic Pathology*, 2d Ed., W.B. Saunders Co., Philadelphia, pp. 68-79.) Hyperplasia is a form of controlled cell proliferation involving an increase in cell number in a tissue or organ, without significant alteration in structure or function. As but one example, endometrial hyperplasia often precedes endometrial cancer. Metaplasia is a form of controlled cell growth in which one type of adult or fully differentiated cell substitutes for another type of adult cell. Metaplasia can occur in epithelial or connective tissue cells. Atypical metaplasia involves a somewhat disorderly metaplastic epithelium. Dysplasia is frequently a forerunner of cancer, and is found mainly in the epithelia; it is the most disorderly form of non-neoplastic cell growth, involving a loss in individual cell uniformity and in the architectural orientation of cells. Dysplastic cells often have abnormally large, deeply stained nuclei, and exhibit pleomorphism. Dysplasia characteristically occurs where there exists chronic irritation or inflammation, and is often found in the cervix, respiratory passages, oral cavity, and gall bladd r.

Alt rnativ ly or in addition to the presence of abnormal cell growth characterized as hyperplasia, metaplasia, or dysplasia, the presence of one or more characteristics of a transformed phenotype, or of a malignant phenotype, displayed *in vivo* or displayed *in vitro* by a cell sample from a patient, can indicate the desirability of prophylactic/therapeutic administration of a Therapeutic that promotes lats function. As mentioned *supra*, such characteristics of a transformed phenotype include morphology changes, looser substratum attachment, loss of contact inhibition, loss of anchorage dependence, protease release, increased sugar transport, decreased serum requirement, expression of fetal antigens, disappearance of the 250,000 dalton cell surface protein, etc. (see also *id.*, at pp. 84-90 for characteristics associated with a transformed or malignant phenotype).

In a specific embodiment, leukoplakia, a benign-appearing hyperplastic or dysplastic lesion of the epithelium, or Bowen's disease, a carcinoma *in situ*, are pre-neoplastic lesions indicative of the desirability of prophylactic intervention.

In another embodiment, fibrocystic disease (cystic hyperplasia, mammary dysplasia, particularly adenosis (benign epithelial hyperplasia)) is indicative of the desirability of prophylactic intervention.

In other embodiments, a patient which exhibits one or more of the following predisposing factors for malignancy is treated by administration of an effective amount of a Therapeutic: a chromosomal translocation associated with a malignancy (*e.g.*, the Philadelphia chromosome for chronic myelogenous leukemia, t(14;18) for follicular lymphoma, etc.), familial polyposis or Gardner's syndrome (possible forerunners of colon cancer), benign monoclonal gammopathy (a possible forerunner of multiple myeloma), and a first degree kinship with persons having a cancer or precancerous disease showing a M ndelian (genetic) inheritance pattern (*e.g.*, familial polyposis of the colon, Gardner's syndrome,

hereditary xostosis, poly ndocrine ad nomatosis, medullary thyroid carcinoma with amyloid production and phe chromocytoma, Peutz-Jeghers syndrome, neurofibromatosis of Von Recklinghausen, retinoblastoma, carotid body tumor, cutaneous melanocarcinoma, intraocular melanocarcinoma, xeroderma pigmentosum, ataxia telangiectasia, Chediak-Higashi syndrome, albinism, Fanconi's aplastic anemia, and Bloom's syndrome; *see* Robbins and Angell, 1976, *Basic Pathology*, 2d Ed., W.B. Saunders Co., Philadelphia, pp. 112-113) etc.)

In another specific embodiment, a Therapeutic of the invention is administered to a human patient to prevent progression to breast, colon, lung, pancreatic, or uterine cancer, or melanoma or sarcoma.

5.8.1.3. HYPERPROLIFERATIVE AND DYSPROLIFERATIVE DISORDERS

In another embodiment of the invention, a Therapeutic that promotes lats activity is used to treat or prevent hyperproliferative or benign dysproliferative disorders. Specific embodiments are directed to treatment or prevention of cirrhosis of the liver (a condition in which scarring has overtaken normal liver regeneration processes), treatment of keloid (hypertrophic scar) formation (disfiguring of the skin in which the scarring process interferes with normal renewal), psoriasis (a common skin condition characterized by excessive proliferation of the skin and delay in proper cell fate determination), benign tumors, fibrocystic conditions, and tissue hypertrophy (*e.g.*, prostatic hyperplasia).

5.8.1.4. GENE THERAPY

In a specific embodiment, nucleic acids comprising a sequence encoding a lats protein or functional derivative thereof, are administered to promote lats function, by way of gene therapy. Gene therapy refers to therapy performed by the administration of a nucleic acid to a subject. In this embodiment of the invention, the nucleic acid produces its

encoded prot in that mediates a therapeutic effect by promoting lats function.

Any of the m thods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel et al., 1993, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIBTECH 11(5):155-215). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

In a preferred aspect, the Therapeutic comprises a lats nucleic acid that is part of an expression vector that expresses a lats protein or fragment or chimeric protein thereof in a suitable host. In particular, such a nucleic acid has a promoter operably linked to the lats coding region, said promoter being inducible or constitutive, and, optionally, tissue-specific. In another particular embodiment, a nucleic acid molecule is used in which the lats coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the lats nucleic acid (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438).

Delivery of the nucleic acid into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vector, or indirect, in which case, cells are first transformed with the nucleic acid *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gen therapy.

In a specific embodiment, the nucleic acid is directly administer d *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, *e.g.*, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by infection using a defective or attenuated retroviral or other viral vector (see U.S. Patent No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in liposomes, microparticles, or microcapsules, or by administering it in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see e.g., Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432) (which can be used to target cell types specifically expressing the receptors), etc. In another embodiment, a nucleic acid-ligand complex can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, e.g., PCT Publications WO 92/06180 dated April 16, 1992 (Wu et al.); WO 92/22635 dated December 23, 1992 (Wilson et al.); WO92/20316 dated November 26, 1992 (Findeis et al.); WO93/14188 dated July 22, 1993 (Clarke et al.), WO 93/20221 dated October 14, 1993 (Young)). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438).

In a specific embodiment, a viral vector that contains the lats nucleic acid is used. For example, a retroviral vector can be used (see Miller et al., 1993, Meth. Enzymol. 217:581-599). These retroviral vectors have been

modified to del te retroviral sequences that are not n c ssary for packaging of the viral genome and integration into host cell DNA. The lats nucleic acid to be used in gene therapy is cloned into the vector, which facilitates delivery of the gene into a patient. More detail about retroviral vectors can be found in Boesen et al., 1994, Biotherapy 6:291-302, which describes the use of a retroviral vector to deliver the mdr1 gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes et al., 1994, J. Clin. Invest. 93:644-651; Kiem et al., 1994, Blood 83:1467-1473; Salmons and Gunzberg, 1993, Human Gene Therapy 4:129-141; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3:110-114.

Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, 1993, Current Opinion in Genetics and Development 3:499-503 present a review of adenovirus-based gene therapy. Bout et al., 1994, Human Gene Therapy 5:3-10 demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld et al., 1991, Science 252:431-434; Rosenfeld et al., 1992, Cell 68:143-155; and Mastrangeli et al., 1993, J. Clin. Invest. 91:225-234.

Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh et al., 1993, Proc. Soc. Exp. Biol. Med. 204:289-300.

Another approach to gene therapy involves transferring a gene to cells in tissue culture by such

methods as el ctroporation, lipof ction, calcium phosphate m diated transfection, or viral infection. Usually, the m thod of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

In this embodiment, the nucleic acid is introduced into a cell prior to administration *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (see *e.g.*, Loeffler and Behr, 1993, Meth. Enzymol. 217:599-618; Cohen et al., 1993, Meth. Enzymol. 217:618-644; Cline, 1985, Pharmac. Ther. 29:69-92) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a patient by various methods known in the art. In a preferred embodiment, epithelial cells are injected, *e.g.*, subcutaneously. In another embodiment, recombinant skin cells may be applied as a skin graft onto the patient. Recombinant blood cells (*e.g.*, hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any d sired, available c ll type, and includ but ar not limited to epithelial c lls, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc.

In a preferred embodiment, the cell used for gene therapy is autologous to the patient.

In an embodiment in which recombinant cells are used in gene therapy, a lats nucleic acid is introduced into the cells such that it is expressible by the cells or their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained *in vitro* can potentially be used in accordance with this embodiment of the present invention. Such stem cells include but are not limited to hematopoietic stem cells (HSC), stem cells of epithelial tissues such as the skin and the lining of the gut, embryonic heart muscle cells, liver stem cells (PCT Publication WO 94/08598, dated April 28, 1994), and neural stem cells (Stemple and Anderson, 1992, Cell 71:973-985).

Epithelial stem cells (ESCs) or keratinocytes can be obtained from tissues such as the skin and the lining of the gut by known procedures (Rheinwald, 1980, Meth. Cell Bio. 21A:229). In stratified epithelial tissue such as the skin, renewal occurs by mitosis of stem cells within the germinal layer, the layer closest to the basal lamina. Stem cells within the lining of the gut provide for a rapid renewal rate of this tissue. ESCs or keratinocytes obtained from the skin or lining of the gut of a patient or donor can be grown in tissue culture (Rh inwald, 1980, M th. Cell Bio. 21A:229; Pittelkow and Scott, 1986, Mayo Clinic Proc. 61:771). If the

ESCs are provid d by a donor, a method for suppression of host versus graft r activity (*e.g.*, irradiation, drug or antibody administration to promote moderate immunosuppression) can also b used.

With respect to hematopoietic stem cells (HSC), any technique which provides for the isolation, propagation, and maintenance *in vitro* of HSC can be used in this embodiment of the invention. Techniques by which this may be accomplished include (a) the isolation and establishment of HSC cultures from bone marrow cells isolated from the future host, or a donor, or (b) the use of previously established long-term HSC cultures, which may be allogeneic or xenogeneic. Non-autologous HSC are used preferably in conjunction with a method of suppressing transplantation immune reactions of the future host/patient. In a particular embodiment of the present invention, human bone marrow cells can be obtained from the posterior iliac crest by needle aspiration (see, *e.g.*, Kodo et al., 1984, J. Clin. Invest. 73:1377-1384). In a preferred embodiment of the present invention, the HSCs can be made highly enriched or in substantially pure form. This enrichment can be accomplished before, during, or after long-term culturing, and can be done by any techniques known in the art. Long-term cultures of bone marrow cells can be established and maintained by using, for example, modified Dexter cell culture techniques (Dexter et al., 1977, J. Cell Physiol. 91:335) or Witlock-Witte culture techniques (Witlock and Witte, 1982, Proc. Natl. Acad. Sci. USA 79:3608-3612).

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

Additional methods that can be adapted for use to deliver a nucleic acid encoding a lats protein or functional derivative thereof ar described in Section 5.8.2.2.2.

5.8.2. TREATMENT AND PREVENTION OF DISORDERS IN WHICH CELL PROLIFERATION IS DESIRED

Diseas s and disorders involving a defici ncy in cell proliferation (growth) or in which cell proliferation is otherwise desirable for treatment or prevention, are treated or prevented by administration of a Therapeutic that antagonizes (inhibits) lats function (in particular, lats-mediated inhibition of cell proliferation). Therapeutics that can be used include but are not limited to anti-lats antibodies (and fragments and derivatives thereof containing the binding region thereof), lats derivatives or analogs that are dominant-negative kinases, *lats* antisense nucleic acids, and lats nucleic acids that are dysfunctional (*e.g.*, due to a heterologous (non-*lats* sequence) insertion within the *lats* coding sequence) that are used to "knockout" endogenous *lats* function by homologous recombination (see, *e.g.*, Capecchi, 1989, Science 244:1288-1292). In a specific embodiment of the invention, a nucleic acid containing a portion of a *lats* gene in which *lats* sequences flank (are both 5′ and 3′ to) a different gene sequence, is used, as a lats antagonist, to promote *lats* inactivation by homologous recombination (see also Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438). Other Therapeutics that inhibit lats function can be identified by use of known convenient *in vitro* assays, *e.g.*, based on their ability to inhibit binding of lats to another protein (*e.g.*, an SH3-domain containing protein), or inhibit any known lats function, as preferably assayed *in vitro* or in cell culture, although genetic assays (*e.g.*, in *Drosophila*) may also be employed. Preferably, suitable *in vitro* or *in vivo* assays, are utilized to determine the effect of a specific Therapeutic and whether its administration is indicated for treatment of the affected tissue.

In specific embodiments, Therapeutics that inhibit lats function are administered therapeutically (including prophylactically): (1) in diseases or disorders involving an increased (relative to normal or desired) level of lats

protein or function, for xampl , in patients where lats prot in is overactive or overexpressed; or (2) in diseases or disorders wherein *in vitro* (or *in vivo*) assays (see *infra*) indicate the utility of lats antagonist administration. The increased levels in lats protein or function can be readily detected, *e.g.*, by quantifying protein and/or RNA, by obtaining a patient tissue sample (*e.g.*, from biopsy tissu) and assaying it *in vitro* for RNA or protein levels, structure and/or activity of the expressed lats RNA or protein. Many methods standard in the art can be thus employed, including but not limited to kinase assays, immunoassays to detect and/or visualize lats protein (*e.g.*, Western blot, immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunocytochemistry, etc.) and/or hybridization assays to detect lats expression by detecting and/or visualizing respectively lats mRNA (*e.g.*, Northern assays, dot blots, *in situ* hybridization, etc.), etc.

Diseases and disorders involving a deficiency in cell proliferation or in which cell proliferation is desired for treatment or prevention, and that can be treated or prevented by inhibiting lats function, include but are not limited to degenerative disorders, growth deficiencies, hypoproliferative disorders, physical trauma, lesions, and wounds; for example, to promote wound healing, or to promote regeneration in degenerated, lesioned or injured tissues, etc. In a specific embodiment, nervous system disorders are treated. In another specific embodiment, a disorder that is not of the nervous system is treated.

Lesions which may be treated according to the present invention include but are not limited to the following lesions:

(i) traumatic lesions, including lesions caused by physical injury or associated with surgery;

(ii) ischemic lesions, in which a lack of oxygen results in cell injury or death, *e.g.*,

myocardial or cerebral infarction or ischemia, or spinal cord infarction or ischemia;

(iii) malignant lesions, in which cells ar destroyed or injured by malignant tissue;

(iv) infectious lesions, in which tissue is destroyed or injured as a result of infection, for example, by an abscess or associated with infection by human immunodeficiency virus, herpes zoster, or herpes simplex virus or with Lyme disease, tuberculosis, syphilis;

(v) degenerative lesions, in which tissue is destroyed or injured as a result of a degenerative process, including but not limited to nervous system degeneration associated with Parkinson's disease, Alzheimer's disease, Huntington's chorea, or amyotrophic lateral sclerosis;

(vi) lesions associated with nutritional diseases or disorders, in which tissue is destroyed or injured by a nutritional disorder or disorder of metabolism including but not limited to, vitamin B12 deficiency, folic acid deficiency, Wernicke disease, tobacco-alcohol amblyopia, Marchiafava-Bignami disease (primary degeneration of the corpus callosum), and alcoholic cerebellar degeneration;

(vii) lesions associated with systemic diseases including but not limited to diabetes or systemic lupus erythematosus;

(viii) lesions caused by toxic substances including alcohol, lead, or other toxins; and

(ix) demyelinated lesions of the nervous system, in which a portion of the nervous system is destroyed or injured by a demyelinating disease including but not limited to multiple sclerosis, human immunodeficiency virus-associated myelopathy, transverse myelopathy

or various tiologi s, progressive multifocal l ukoencephalopathy, and central pontine my linolysis.

Nervous system lesions which may be treated in a patient (including human and non-human mammalian patients) according to the invention include but are not limited to the lesions of either the central (including spinal cord, brain) or peripheral nervous systems.

Therapeutics which are useful according to this embodiment of the invention for treatment of a disorder may be selected by testing for biological activity in promoting the survival or differentiation of cells (see also Section 5.9). For example, in a specific embodiment relating to therapy of the nervous system, a Therapeutic which elicits one of the following effects may be useful according to the invention:

(i) increased sprouting of neurons in culture or *in vivo*;

(ii) increased production of a neuron-associated molecule in culture or *in vivo*, *e.g.*, cholin acetyltransferase or acetylcholinesterase with respect to motor neurons; or

(iii) decreased symptoms of neuron dysfunction *in vivo*.

Such effects may be measured by any method known in the art. In preferred, non-limiting embodiments, increased sprouting of neurons may be detected by methods set forth in Pestronk et al. (1980, Exp. Neurol. 70:65-82) or Brown et al. (1981, Ann. Rev. Neurosci. 4:17-42); and increased production of neuron-associated molecules may be measured by bioassay, enzymatic assay, antibody binding, Northern blot assay, etc., depending on the molecule to be measured.

5.8.2.1. <u>ANTISENSE REGULATION OF *LATS* EXPRESSION</u>

In a specific embodiment, lats function is inhibit d by us of *lats* antisense nucleic acids. The

pres nt invention provides the therapeutic or prophylactic us of nucleic acids of at least six nucleotides that are antis nse to a gene or cDNA encoding lats or a portion thereof. A *lats* "antisense" nucleic acid as used herein refers to a nucleic acid capable of hybridizing to a portion of a *lats* RNA (preferably mRNA) by virtue of some sequence complementarity. The antisense nucleic acid may be complementary to a coding and/or noncoding region of a *lats* mRNA. Such antisense nucleic acids have utility as Therapeutics that inhibits lats function, and can be used in the treatment or prevention of disorders as described *supra* in Section 5.8.2 and its subsections.

The antisense nucleic acids of the invention can be oligonucleotides that are double-stranded or single-stranded, RNA or DNA or a modification or derivative thereof, which can be directly administered to a cell, or which can be produced intracellularly by transcription of exogenous, introduced sequences.

In a specific embodiment, the *lats* antisense nucleic acids provided by the instant invention can be used to promote regeneration or wound healing or to promote growth (larger size).

The invention further provides pharmaceutical compositions comprising an effective amount of the *lats* antisense nucleic acids of the invention in a pharmaceutically acceptable carrier, as described *infra*.

In another embodiment, the invention is directed to methods for inhibiting the expression of a *lats* nucleic acid sequence in a prokaryotic or eukaryotic cell comprising providing the cell with an effective amount of a composition comprising an *lats* antisense nucleic acid of the invention.

Lats antisense nucleic acids and their uses are described in detail below.

5.8.2.1.1. *LATS* ANTISENSE NUCLEIC ACIDS

The *lats* antisense nucleic acids are of at least six nucleotides and are preferably oligonucleotides (ranging from 6 to about 50 oligonucleotides). In specific aspects, the oligonucleotide is at least 10 nucleotides, at least 15 nucleotides, at least 100 nucleotides, or at least 200 nucleotides. The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may include other appending groups such as peptides, or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556; Lemaitre et al., 1987, Proc. Natl. Acad. Sci. 84:648-652; PCT Publication No. WO 88/09810, published December 15, 1988) or blood-brain barrier (see, *e.g.*, PCT Publication No. WO 89/10134, published April 25, 1988), hybridization-triggered cleavage agents (see, *e.g.*, Krol et al., 1988, BioTechniques 6:958-976) or intercalating agents (see, *e.g.*, Zon, 1988, Pharm. Res. 5:539-549).

In a preferred aspect of the invention, a *lats* antisense oligonucleotide is provided, preferably of single-stranded DNA. In a most preferred aspect, such an oligonucleotide comprises a sequence antisense to the sequence encoding an SH3 binding domain or a kinase domain of a lats protein, most preferably, of a human lats protein. The oligonucleotide may be modified at any position on its structure with substituents generally known in the art.

The lats antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, in sine, N6-isopentenyladenine,

1-methylguanine, 1-methylin sine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-m thylcytosin , N6-adenine, 7-methylguanine, 5-m thylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

In another embodiment, the oligonucleotide comprises at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the oligonucleotide is an α-anomeric oligonucleotide. An α-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641).

The oligonucleotide may be conjugated to another molecule, *e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

Oligonucleotides of the invention may be synthesized by standard methods known in the art, *e.g.* by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be

synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), methylphosphonate oligonucleotides can be pr pared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

In a specific embodiment, the *lats* antisense oligonucleotide comprises catalytic RNA, or a ribozyme (see, *e.g.*, PCT International Publication WO 90/11364, published October 4, 1990; Sarver et al., 1990, Science 247:1222-1225). In another embodiment, the oligonucleotide is a 2'-0-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330).

In an alternative embodiment, the *lats* antisense nucleic acid of the invention is produced intracellularly by transcription from an exogenous sequence. For example, a vector can be introduced *in vivo* such that it is taken up by a cell, within which cell the vector or a portion thereof is transcribed, producing an antisense nucleic acid (RNA) of the invention. Such a vector would contain a sequence encoding the *lats* antisense nucleic acid. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the lats antisense RNA can be by any promoter known in the art to act in mammalian, preferably human, cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the

regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42), etc.

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a *lats* gene, preferably a human *lats* gene. However, absolute complementarity, although preferred, is not required. A sequence "complementary to at least a portion of an RNA," as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded lats antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with a *lats* RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

5.8.2.1.2. THERAPEUTIC USE OF *LATS* ANTISENSE NUCLEIC ACIDS

The *lats* antisense nucleic acids can be used to treat (or prevent) disorders of a cell type that expresses, or preferably overexpresses, *lats*. In a specific embodiment, such a disorder is a growth deficiency. In a preferred embodiment, a single-stranded DNA antisense lats oligonucleotide is used.

Cell types which express or overexpress *lats* RNA can be identified by various methods known in the art. Such methods include but are not limited to hybridization with a *lats*-specific nucleic acid (*e.g.* by Northern hybridization, dot blot hybridization, *in situ* hybridization), observing the ability of RNA from the cell type to be translated *in vitro* into lats, immunoassay, etc. In a preferred aspect, primary tissue from a patient can be assayed for lats expression

prior to treatment, *e.g.*, by immunocytochemistry or *in situ* hybridization.

Pharmaceutical compositions of th inv ntion (see Section 5.10), comprising an effective amount of a *lats* antisense nucleic acid in a pharmaceutically acceptable carrier, can be administered to a patient having a disease or disorder which is of a type that expresses or overexpresses *lats* RNA or protein.

The amount of *lats* antisense nucleic acid which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. Where possible, it is desirable to determine the antisense cytotoxicity of the tumor type to be treated *in vitro*, and then in useful animal model systems prior to testing and use in humans.

In a specific embodiment, pharmaceutical compositions comprising *lats* antisense nucleic acids are administered via liposomes, microparticles, or microcapsules. In various embodiments of the invention, it may be useful to use such compositions to achieve sustained release of the *lats* antisense nucleic acids. In a specific embodiment, it may be desirable to utilize liposomes targeted via antibodies to specific identifiable tumor antigens (Leonetti et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:2448-2451; Renneisen et al., 1990, J. Biol. Chem. 265:16337-16342).

Additional methods that can be adapted for use to deliver a *lats* antisense nucleic acid are described in Section 5.8.1.4.

5.9. DEMONSTRATION OF THERAPEUTIC OR PROPHYLACTIC UTILITY

The Therapeutics of the invention are preferably tested *in vitro*, and then *in vivo* for the desired therapeutic or prophylactic activity, prior to use in humans. For example, *In vitro* assays which can be used to determine whether administration of a specific Therapeutic is

indicated, include *in vitro* cell culture assays in which a patient tissue sample is grown in culture, and expos d to or otherwis administered a Therapeutic, and the effect of such Therapeutic upon the tissue sample is observed. In one embodiment, where the patient has a malignancy, a sample of cells from such malignancy is plated out or grown in culture, and the cells are then exposed to a Therapeutic. A Therapeutic which inhibits survival or growth of the malignant cells is selected for therapeutic use *in vivo*. Many assays standard in the art can be used to assess such survival and/or growth; for example, cell proliferation can be assayed by measuring ^{3}H-thymidine incorporation, by direct cell count, by detecting changes in transcriptional activity of known genes such as proto-oncogenes (*e.g.*, *fos*, *myc*) or cell cycle markers; cell viability can be assessed by trypan blue staining, differentiation can be assessed visually based on changes in morphology, etc.

In another embodiment, a Therapeutic is indicated for use which exhibits the desired effect, inhibition or promotion of cell growth, upon a patient cell sample from tissue having or suspected of having a hyper- or hypoproliferative disorder, respectively. Such hyper- or hypoproliferative disorders include but are not limited to those described in Sections 5.8.1 through 5.8.3 *infra*.

In another specific embodiment, a Therapeutic is indicated for use in treating cell injury or a degenerative disorder (see Section 5.8.2) which exhibits *in vitro* promotion of growth/proliferation of cells of the affected patient type. Regarding nervous system disorders, see also Section 5.8.2.1 for assays that can be used.

In various specific embodiments, *in vitro* assays can be carried out with representative cells of cell types involved in a patient's disorder, to determine if a Therapeutic has a desired effect upon such cell types.

In another embodiment, cells of a patient tissue sample suspected of being pre-neoplastic are similarly plated out or grown *in vitro*, and exposed to a Therapeutic. The

Therapeutic which results in a cell phenotype that is more normal (*i. .*, l ss representative of a pre-neoplastic stat , n oplastic state, malignant state, or transformed phenotyp) is selected for therapeutic us . Many assays standard in the art can be used to assess whether a pre-neoplastic state, neoplastic state, or a transformed or malignant phenotype, is present. For example, characteristics associated with a transformed phenotype (a set of *in vitro* characteristics associated with a tumorigenic ability *in vivo*) include a more rounded cell morphology, looser substratum attachment, loss of contact inhibition, loss of anchorage dependence, release of proteases such as plasminogen activator, increased sugar transport, decreased serum requirement, expression of fetal antigens, disappearance of the 250,000 dalton surface protein, etc. (see Luria et al., 1978, *General Virology*, 3d Ed., John Wiley & Sons, New York pp. 436-446).

In other specific embodiments, the *in vitro* assays described *supra* can be carried out using a cell line, rather than a cell sample derived from the specific patient to be treated, in which the cell line is derived from or displays characteristic(s) associated with the malignant, neoplastic or pre-neoplastic disorder desired to be treated or prevented, or is derived from the cell type upon which an effect is desired, according to the present invention.

Compounds for use in therapy can be tested in suitable animal model systems prior to testing in humans, including but not limited to rats, mice, chicken, cows, monkeys, rabbits, etc. For *in vivo* testing, prior to administration to humans, any animal model system known in the art may be used.

5.10. THERAPEUTIC/PROPHYLACTIC ADMINISTRATION AND COMPOSITIONS

The invention provides methods of treatment (and prophylaxis) by administration to a subject of an effective amount of a Th rap utic of the invention. In a preferred aspect, the Therapeutic is substantially purified. The

subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, tc., and is pref rably a mammal, and most preferably human. In a sp cific embodiment, a non-human mammal is the subject.

Formulations and methods of administration that can be employed when the Therapeutic comprises a nucleic acid are described in Sections 5.8.1.4 and 5.8.2.2 above; additional appropriate formulations and routes of administration can be selected from among those described hereinbelow.

Various delivery systems are known and can be used to administer a Therapeutic of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the Therapeutic, receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), construction of a Therapeutic nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmac utical compositions of the invention locally to the area in need of treatment; this may be

achi ved by, for xample, and not by way of limitation, local infusion during surgery, topical application, *e.g.*, in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

In another embodiment, the Therapeutic can be delivered in a vesicle, in particular a liposome (*see* Langer, Science 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; *see* generally *ibid.*)

In yet another embodiment, the Therapeutic can be delivered in a controlled release system. In one embodiment, a pump may be used (*see* Langer, *supra*; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)). In another embodiment, polymeric materials can be used (*see* Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J. Macromol. Sci. Rev. Macromol. Chem. 23:61 (1983); *see also* Levy et al., Science 228:190 (1985); During et al., Ann. Neurol. 25:351 (1989); Howard et al., J. Neurosurg. 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, *i.e.*, the brain, thus requiring only a fraction of the systemic dose (*see*, *e.g.*, Goodson, in Medical Applications of Controlled Release, *supra*, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (Science 249:1527-1533 (1990)).

In a specific embodiment where the Therapeutic is a nucleic acid encoding a protein Therapeutic, the nucleic acid can b administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see *e.g.*, Joliot et al., 1991, Proc. Natl. Acad. Sci. USA 88:1864-1868), etc. Alternatively, a nucleic acid Therapeutic can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a Therapeutic, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium

chlorid , dri d skim milk, glycerol, propyl ne, glycol, water, ethanol and th like. The composition, if desired, can also contain minor amounts of wetting or emulsifying ag nts, or pH buff ring agents. These comp sitions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the Therapeutic, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mix d prior to administration.

The Therap utics of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ~~ammonium, calcium,~~ ferr~~ic~~ hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the Therapeutic of the invention which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmac uticals or biological products, which notice reflects

approval by the agency of manufacture, use or sale for human administration.

5.11. ADDITIONAL USE OF INHIBITION OF LATS FUNCTION TO PROMOTE INCREASED GROWTH

Inhibition of lats function (*e.g.*, by administering a compound that inhibits lats function as described in Sections 5.8.2 through 5.8.2.1.2 above), has utility that is not limited to therapeutic or prophylactic applications. For example, lats function can be inhibited in order to increase growth of animals (*e.g.*, cows, horses, pigs, goats, deer, chickens) and plants (particularly edible plants, *e.g.*, tomatoes, melons, lettuce, carrots, potatoes, and other vegetables), particularly those that are food or material sources. For example, antisense inhibition (preferably where the lats antisense nucleic acid is under the control of a tissue-specific promoter) can be used in plants or animals to increase growth where desired (*e.g.*, in the fruit or muscle). For example, a *lats* antisense nucleic acid under the control of a temperature-sensitive promoter can be administered to a plant or animal, and the desired portion of the (or the entire) plant or animal can be subjected to heat in order to induce antisense nucleic acid production, resulting lats inhibition, and resulting cell proliferation. In other embodiments, chemical mutagenesis, or homologous recombination with an insertionally inactivated lats gene (see Capecchi, 1989, Science 244:1288-1292 and Section 5.14 *infra*) can be carried out to reduce or destroy endogenous lats function, in order to achieve increased growth. Suitable methods, modes of administration and compositions, that can be used to inhibit lats function are described in Sections 5.8.2 through 5.8.2.1.2, above. Methods to make plants recombinant are commonly known in the art and can be used. Regarding methods of plant transformation (*e.g.*, for transformation with a *lats* antisense nucleic acid or with a sequence encoding a lats derivative that is a dominant-negative kinase), see *e.g.*, Valvek ns et al., 1988, Proc.

Natl. Acad. Sci. USA 85:5536-5540. Regarding methods of targeted gene inactivation in plants (*e.g.*, to inactivate *lats*), see *e.g.*, Miao and Lam, 1995, The Plant J. 7:359-365.

Inhibition of lats function can also have uses *in vitro*, *e.g.*, to expand cells *in vitro*, including but not limited to stem cells, progenitor cells, muscle cells, fibroblasts, liver cells, etc., *e.g.*, to grow cells/tissue *in vitro* prior to administration to a patient (preferably a patient from which the cells were derived), etc.

5.12. ADDITIONAL USE OF INHIBITION OF LATS FUNCTION TO INHIBIT CELLULAR SENESCENCE

Inhibition of lats function (*e.g.*, by administering a compound that inhibits lats function as described in Sections 5.8.2 through 5.8.2.1.2 above), also has utility in the inhibition of cellular senescence. Thus, inhibition of lats function can be carried out to delay or prevent the onset of cellular senescence, *in vivo* or *in vitro*. In a specific embodiment, cellular senescence is delayed or prevented without incurring the onset of cell malignancy or its *in vitro* correlate, a transformed phenotype.

Thus, for example, a lats antagonist (*e.g.*, anti-lats antibody, lats derivatives or analogs that are dominant-negative kinases; lats antisense nucleic acids, etc.) can be administered to a subject to inhibit or prevent aging or cell death or the effects of aging or cell death (*e.g.*, in the skin, wrinkling, loss of elasticity, less uniform skin tone; in the skin and elsewhere, loss of known characteristics of proper physiological function such as expression of characteristic antigens, secreted molecules, etc.) In one embodiment, a lats antagonist is applied topically, *e.g.*, in a cream or gel, to the skin of the subject. In another embodiment, a lats antagonist is injected, *e.g.*, intradermally, intraperitoneally, or intramuscularly.

In a specific embodiment, a lats antagonist is contacted with cells grown in cultur , *e.g.*, by addition of the antagonist to the culture medium or by adsorption of the

antagonist to the culture plate or flask prior to seeding of the cells, in ord r to inhibit or d lay senescence *in vitro*, *e.g.*, to delay "crisis" phase. For example, such a method can b carried out in ord r to lengthen the time that cells can be kept alive *in vitro*, *e.g.*, in order to facilitate conducting studies of the toxicity of a compound (*e.g.*, a lead drug candidate) upon such cells, to study the effect of a molecule upon cell function, and, generally, to study the function of such cells. Such cells include but are not limited to neurons of the central nervous system (*e.g.*, hippocampal, hypothalmic) or peripheral nervous system, glial cells, fibroblasts, kidney cells, liver cells, heart cells, muscle cells, endothelial cells, melanocytes, and hematopoietic cells such and T and B lymphocytes, macrophages, granulocytes, and mast cells.

In vitro assays of senescence are well known in the art and can be used to screen potential lats antagonists prior to use in this aspect of the invention (*see, e.g.*, Hubbard and Ozer, 1995, "Senescence and immortalization of human cells," in <u>Cell Growth and Apoptosis, A Practical Approach</u>, Ch. 12, Studzinski, G.P. (ed.), Oxford University Press, Inc., New York, NY, pp. 229-248.

5.13. <u>DIAGNOSIS AND SCREENING</u>

Lats proteins, analogues, derivatives, and subsequences thereof, *lats* nucleic acids (and sequences complementary thereto), anti-lats antibodies, have uses in diagnostics. Such molecules can be used in assays, such as immunoassays, to detect, prognose, diagnose, or monitor various conditions, diseases, and disorders affecting lats expression, or monitor the treatment thereof. In particular, such an immunoassay is carried out by a method comprising contacting a sample derived from a patient with an anti-lats antibody under conditions such that immunospecific binding can occur, and detecting or measuring the amount of any immunospecific binding by th antibody. In a specific aspect, such binding of antibody, in tissue sections, can be

used to detect ab rrant lats localization or aberrant (*e.g.*, low or absent) lev ls of lats. In a specific embodiment, antibody to lats can be used to assay in a patient tissue or s rum sample for the presence of lats where an aberrant level of lats is an indication of a diseased condition. By "aberrant levels," is meant increased or decreased levels relative to that present, or a standard level representing that present, in an analogous sample from a portion of the body or from a subject not having the disorder.

The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few.

Lats genes and related nucleic acid sequences and subsequences, including complementary sequences, can also be used in hybridization assays. *Lats* nucleic acid sequences, or subsequences thereof comprising about at least 8 nucleotides, can be used as hybridization probes. Hybridization assays can be used to detect, prognose, diagnose, or monitor conditions, disorders, or disease states associated with aberrant changes in lats expression and/or activity as described *supra*. In particular, such a hybridization assay is carried out by a method comprising contacting a sample containing nucleic acid with a nucleic acid probe capable of hybridizing to *lats* DNA or RNA, under conditions such that hybridization can occur, and detecting or measuring any resulting hybridization.

In specific embodiments, diseases and disorders involving overproliferation of cells can be diagnosed, or their suspected presence can be screened for, or a predisposition to develop such disorders can be detected, by detecting decreased levels of lats protein, lats RNA, or lats

functional activity (*e.g.*, kinase activity, SH3 domain-binding activity, tc.), or by detecting mutations in lats RNA, DNA or protein (*e.g.*, translocations in lats nucleic acids, truncations in the lats gene or protein, changes in nucleotide or amino acid sequence relative to wild-type lats) that cause decreased expression or activity of lats. Such diseases and disorders include but are not limited to those described in Section 5.8.1 and its subsections. By way of example, levels of lats protein can be detected by immunoassay, levels of lats RNA can be detected by hybridization assays (*e.g.*, Northern blots, dot blots), lats kinase activity can be measured by kinase assays commonly known in the art, lats binding to an SH3 domain-containing protein can be done by binding assays commonly known in the art, translocations and point mutations in lats nucleic acids can be detected by Southern blotting, RFLP analysis, PCR using primers that preferably generate a fragment spanning at least most of the lats gene, sequencing of the lats genomic DNA or cDNA obtained from the patient, etc.

In a preferred embodiment, levels of lats mRNA or protein in a patient sample are detected or measured, in which decreased levels indicate that the subject has, or has a predisposition to developing, a malignancy or hyperproliferative disorder; in which the decreased levels are relative to the levels present in an analogous sample from a portion of the body or from a subject not having the malignancy or hyperproliferative disorder, as the case may be.

In another specific embodiment, diseases and disorders involving a deficiency in cell proliferation or in which cell proliferation is desirable for treatment, are diagnosed, or their suspected presence can be screened for, or a predisposition to develop such disorders can be detected, by detecting increased levels of lats protein, lats RNA, or lats functional activity (*e.g.*, kinase activity, SH3 domain binding activity, etc.), or by detecting mutations in lats RNA, DNA or protein (*e.g.*, translocations in lats

nucl ic acids, truncations in the gene or protein, changes in nucl otide or amino acid sequence relative to wild-type lats) that cause incr ased expr ssion or activity of lats. Such diseases and disorders include but are not limited to those described in Section 5.8.2 and its subsections. By way of example, levels of lats protein, levels of lats RNA, lats kinase activity, lats binding activity, and the presence of translocations or point mutations can be determined as described above.

In a specific embodiment, levels of lats mRNA or protein in a patient sample are detected or measured, in which increased levels indicate that the subject has, or has a predisposition to developing, a growth deficiency or degenerative or hypoproliferative disorder; in which the increased levels are relative to the levels present in an analogous sample from a portion of the body or from a subject not having the growth deficiency, degenerative, or hypoproliferative disorder, as the case may be.

Kits for diagnostic use are also provided, that comprise in one or more containers an anti-lats antibody, and, optionally, a labeled binding partner to the antibody. Alternatively, the anti-lats antibody can be labeled (with a detectable marker, *e.g.*, a chemiluminescent, enzymatic, fluorescent, or radioactive moiety). A kit is also provided that comprises in one or more containers a nucleic acid probe capable of hybridizing to *lats* RNA. In a specific embodiment, a kit can comprise in one or more containers a pair of primers (*e.g.*, each in the size range of 6-30 nucleotides) that are capable of priming amplification [*e.g.*, by polymerase chain reaction (see *e.g.*, Innis et al., 1990, PCR Protocols, Academic Press, Inc., San Diego, CA), ligase chain reaction (see EP 320,308) use of $Q\beta$ replicase, cyclic probe reaction, or other methods known in the art] under appropriate reaction conditions of at least a portion of a *lats* nucleic acid. A kit can optionally further comprise in a container a predetermined amount of a purified lats protein or nucleic acid, *e.g.*, for use as a standard or control.

5.14. SCREENING FOR LATS AGONISTS AND ANTAGONISTS

Lats nucleic acids, proteins, and derivativ s also have uses in scr ening assays to detect molecules that sp cifically bind to lats nucleic acids, proteins, or derivatives and thus have potential use as agonists or antagonists of lats, in particular, molecules that thus affect cell proliferation. In a preferred embodiment, such assays are performed to screen for molecules with potential utility as anti-cancer drugs or lead compounds for drug development. The invention thus provides assays to detect molecules that specifically bind to lats nucleic acids, proteins, or derivatives. For example, recombinant cells expressing *lats* nucleic acids can be used to recombinantly produce lats proteins in these assays, to screen for molecules that bind to a lats protein. Molecules (*e.g.*, putative binding partners of lats) are contacted with the lats protein (or fragment thereof) under conditions conducive to binding, and then molecules that specifically bind to the lats protein are identified. Similar methods can be used to screen for molecules that bind to lats derivatives or nucleic acids. Methods that can be used to carry out the foregoing are commonly known in the art.

By way of example, diversity libraries, such as random or combinatorial peptide or nonpeptide libraries can be screened for molecules that specifically bind to lats. Many libraries are known in the art that can be used, *e.g.*, chemically synthesized libraries, recombinant (*e.g.*, phage display libraries), and *in vitro* translation-based libraries.

Examples of chemically synthesized libraries are described in Fodor et al., 1991, Science 251:767-773; Houghten et al., 1991, Nature 354:84-86; Lam et al., 1991, Nature 354:82-84; Medynski, 1994, Bio/Technology 12:709-710; Gallop et al., 1994, J. Medicinal Chemistry 37(9):1233-1251; Ohlmeyer et al., 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926; Erb et al., 1994, Proc. Natl. Acad. Sci. USA 91:11422-11426; Houghten et al., 1992, Biotechniques 13:412; Jayawickreme et al., 1994, Proc. Natl. Acad. Sci. USA

91:1614-1618; Salmon t al., 1993, Proc. Natl. Acad. Sci. USA 90:11708-11712; PCT Publication No. WO 93/20242; and Brenner and Lerner, 1992, Proc. Natl. Acad. Sci. USA 89:5381-5383.

Examples of phag display libraries are described in Scott and Smith, 1990, Science 249:386-390; Devlin et al., 1990, Science, 249:404-406; Christian, R.B., et al., 1992, J. Mol. Biol. 227:711-718); Lenstra, 1992, J. Immunol. Meth. 152:149-157; Kay et al., 1993, Gene 128:59-65; and PCT Publication No. WO 94/18318 dated August 18, 1994.

In vitro translation-based libraries include but are not limited to those described in PCT Publication No. WO 91/05058 dated April 18, 1991; and Mattheakis et al., 1994, Proc. Natl. Acad. Sci. USA 91:9022-9026.

By way of examples of nonpeptide libraries, a benzodiazepine library (*see e.g.*, Bunin et al., 1994, Proc. Natl. Acad. Sci. USA 91:4708-4712) can be adapted for use. Peptoid libraries (Simon et al., 1992, Proc. Natl. Acad. Sci. USA 89:9367-9371) can also be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh et al. (1994, Proc. Natl. Acad. Sci. USA 91:11138-11142).

Screening the libraries can be accomplished by any of a variety of commonly known methods. See, *e.g.*, the following references, which disclose screening of peptide libraries: Parmley and Smith, 1989, Adv. Exp. Med. Biol. 251:215-218; Scott and Smith, 1990, Science 249:386-390; Fowlkes et al., 1992; BioTechniques 13:422-427; Oldenburg et al., 1992, Proc. Natl. Acad. Sci. USA 89:5393-5397; Yu et al., 1994, Cell 76:933-945; Staudt et al., 1988, Science 241:577-580; Bock et al., 1992, Nature 355:564-566; Tuerk et al., 1992, Proc. Natl. Acad. Sci. USA 89:6988-6992; Ellington et al., 1992, Nature 355:850-852; U.S. Patent No. 5,096,815, U.S. Patent No. 5,223,409, and U.S. Patent No. 5,198,346, all to Ladner et al.; Rebar and Pabo, 1993, Science 263:671-673; and PCT Publication No. WO 94/18318.

In a specific embodiment, screening can be carried out by contacting the library members with a lats protein (or nucleic acid or derivativ) immobilized on a solid phase and harv sting those library members that bind to the protein (or nucleic acid or derivative). Examples of such screening methods, termed "panning" techniques are described by way of ~~example in Parmley and Smith, 1988,~~ Gene 73:305-318; Fowlkes et al., 1992, BioTechniques 13:422-427; PCT Publication No. WO 94/18318; and in references cited hereinabove.

In another embodiment, the two-hybrid system for selecting interacting proteins in yeast (Fields and Song, 1989, Nature 340:245-246; Chien et al., 1991, Proc. Natl. Acad. Sci. USA 88:9578-9582) can be used to identify molecules that specifically bind to a lats protein or derivative.

In addition, *Drosophila* can be used as a model system in order to detect genes that phenotypically interact with *lats*. For example, overexpression of lats in *Drosophila* eye leads to a smaller and rougher eye. Mutagenesis of the fly genome can be performed, followed by selecting flies in which the mutagenesis has resulted in suppression or enhancement of the small rough eye phenotype; the mutated genes in such flies are likely to encode proteins that interact/bind with lats.

5.15. <u>ANIMAL MODELS</u>

The invention also provides animal models.

In one embodiment, animal models for diseases and disorders involving cell overproliferation (*e.g.*, as described in Section 5.8.1) are provided. Such an animal can be initially produced by promoting homologous recombination between a lats gene in its chromosome and an exogenous lats gene that has been rendered biologically inactive (preferably by insertion of a heterologous sequence, *e.g.*, an antibiotic resistance gene). In a preferred aspect, this homologous recombination is carried out by transforming embryo-derived stem (ES) cells with a vector containing the insertionally

inactivat d lats gene, such that homologous recombination occurs, followed by injecting the ES cells into a blastocyst, and implanting the blastocyst into a foster mother, followed by the birth of the chimeric animal ("knockout animal") in which a lats gene has been inactivated (see Capecchi, 1989, Science 244:1288-1292). The chimeric animal can be bred to produce additional knockout animals. Such animals can be mice, hamsters, sheep, pigs, cattle, etc., and are preferably non-human mammals. In a specific embodiment, a knockout mouse is produced.

Such knockout animals are expected to develop or be predisposed to developing diseases or disorders involving cell overproliferation (*e.g.*, malignancy) and thus can have use as animal models of such diseases and disorders, *e.g.*, to screen for or test molecules (*e.g.*, potential anti-cancer therapeutics) for the ability to inhibit overproliferation (*e.g.*, tumor formation) and thus treat or prevent such diseases or disorders.

In a different embodiment of the invention, transgenic animals that have incorporated and express a functional lats gene have use as animal models of diseases and disorders involving deficiencies in cell proliferation or in which cell proliferation is desired. Such animals can be used to screen for or test molecules for the ability to promote proliferation and thus treat or prevent such diseases and disorders.

5.16. METHODS OF IDENTIFYING TUMOR SUPPRESSOR GENES AND OTHER GENES WITH IDENTIFIABLE PHENOTYPES

The invention also provides methods of identifying a tumor suppressor gene (or potential tumor suppressor gene) comprising identifying an overproliferation phenotype in a genetic mosaic, and isolating a gene that is mutated in cells exhibiting the overproliferation phenotype. The genetic mosaic is achieved by induction of somatic cells in an animal that is heterozygous for an induced mutation to become

homozygous for the mutation, at any desir d developmental stage. Th mutation can be induced by any known method, *e.g.*, X-ray xposure or chemical mutation exposure or insertion of a transposable element (*e.g.*, P-element). A genetic mosaic is produced by induction of homozygosity by mitotic recombination between homologous arms of both parental chromosomes, which is achieved using a site-specific recombination system [a sequence capable of expressing a site-specific recombinase; and its target sites (sequences at which the recombinase promotes recombination)], that have been inserted in the homozygous arms of both parental chromosomes. The target sites are preferably inserted close to the centromere on each chromosome arm (the closer to the centromere, the more preferred), so that mitotic recombination events will result in cells being homozygous for the mutation located on the chromosome arm distal to the insertion of the target site. For example, an FLP recombinase can be used with FRT target sites; Cre recombinase can be used with lox target sites. The recombinase coding sequence, used to express recombinase, preferably, but need not be, intrachromosomally situated. For at least one chromosome, the target sites are intrachromosomally inserted on the homologous arms of both parental (maternal and paternal) chromosomes.

The genetic mosaic can be an animal, *e.g.*, mouse, hamster, sheep, pig, cow, *Drosophila*, etc., and is preferably a non-human mammal.

In a specific embodiment relating to the production of a non-human mammal that is a genetic mosaic, a recombinase target site is introduced onto one arm of a chromosome in an embryo-derived stem cell (ES). The target site can be introduced into the cell by homologous recombination (by use of flanking sequences from the desired site of intrachromosomal integration) or by random integration resulting from cell transformation (*e.g.*, by transfection, electroporation), etc. This ES is then injected into a blastocyst, the blastocyst is implanted into a foster mother,

f llowed by birth of the recombinant animal. This mammal is bred to a wild-typ female, to produce siblings. Siblings carrying the targ t site insertion are mated, and offspring carrying the target site on the homologous arms of both parental chromosomes are isolated ("the target strain"). A target strain member is then mutagenized and mated with a non-mutagenized target strain member of the opposite sex (preferably also carrying a recombinant nucleic acid encoding and capable of expressing a recombinase that promotes recombination at the target sites), to obtain a target strain member that is heterozygous for the mutation. Provision of the recombinase (by expression) in mitotically active cells of a developing animal or an adult animal promotes mitotic recombination between the homologous arms of the parental chromosomes, resulting in a cell that is homozygous for the mutation. Cells that display a mutant phenotype by virtue of their being homozygous for the mutation are then detected, and the mutant gene can be genetically mapped by any known method, and can be isolated.

In a *Drosophila* animal, a site-specific recombination system can be introduced by use of P-element-mediated insertions.

In one embodiment, target sites are introduced onto homologous arms of both of a set of parental chromosomes, for one chromosome. In another embodiment, target sites are introduced onto homologous arms of both of a set of parental chromosomes, for a plurality of chromosomes.

The recombinase can be under the control of a constitutive (*e.g.*, phosphorylated kinase promoter) or inducible (*e.g.*, heat shock promoter) or tissue-specific promoter. The recombinase can be expressed episomally (*e.g.*, from a plasmid) or chromosomally. Once the recombination system is introduced into the animal, genetic mosaicism is produced by the activity of the recombinase (which promotes recombination at the target sites).

In a specific embodiment, an animal is used that contains a recombinant nucl ic acid encoding an FLP

recombinase (Broach and Hicks, 1980, Cell 21:501-508) such that it is expr ssible by a cell of the animal, and intrachromosomal ins rtions of an FRT site on the homologous arms of both parental chromosomes; and genetic mosaicism is produced by inducing mitotic recombination between the FRT sites on the homologous chromosome arms after FLP recombinase expression (*e.g.*, by heat shock, when expression of the FLP recombinase is under the control of a heat shock promoter).

In another specific embodiment, an animal is used that contains a recombinant nucleic acid encoding a Cre recombinase (Sauer and Henderson, 1988, Proc. Natl. Acad. Sci. USA 85:5166-5170) such that it is expressible by a cell of the animal, and intrachromosomal insertions of a lox site on homologous arms of both parental chromosomes; and genetic mosaicism is produced by inducing mitotic recombination between the lox sites on the homologous chromosome arms after Cre recombinase expression.

The animal may optionally further comprise intrachromosomal insertions of marker genes (comprising a sequence encoding a protein containing a reporter group such as an epitope tag), to facilitate confirmation and/or monitoring of recombination events. For example, in a non-human mammal, a marker gene (*e.g.*, *lacZ*) operably linked to a constitutive promoter can be inserted, on the same chromosome arm as that carrying the target site and the induced mutation.

In a specific embodiment, the overproliferation phenotype is the formation of overproliferated outgrowth tissue in a non-position-dependent fashion. In another specific embodiment, the overproliferation phenotype is the formation of a normal structure of larger than normal size.

The above-described genetic mosaics have uses not only in identifying tumor suppressor genes, but, more generally, in identifying genes with an identifiable phenotype, *i.e.*, those genes which in mutated form cause an observable mutant ph notype to be displayed in the genetic mosaic.

In anoth r embodiment, the invention provides a method of identifying genes with an observable mutant phenotyp by us of human (or other animal) tissue culture c lls that have incorporated a site-specific recombination system such as described above. The site-specific recombination system can be introduced by methods such as described above, so as to introduce a recombinant source of recombinase and effect intrachromosomal insertions of the recombinase target sites on the homologous arms of both of a set of parental chromosomes, for one or more chromosomes. In a preferred aspect relating to this use of culture cells, the recombinase target sites are ligated to a selectable marker (*e.g.*, an antibiotic resistance gene), and cells are obtained that have the target sites on each of the homologous chromosome arms, by selecting under selection conditions of relatively high stringency (*e.g.*, by increasing the antibiotic concentration in the cell medium), As with the use of genetic mosaics as described above, once mitotic recombination is induced between the target sites on the homologous chromosome arms, one then identifies cells displaying a mutant phenotype, and recovers a gene mutated in cells exhibiting the mutant phenotype. For example, a potential tumor suppressor gene can be identified by isolating a gene that is mutated in cultured cells exhibiting a transformed phenotype.

6. IDENTIFYING TUMOR SUPPRESSORS IN GENETIC MOSAICS: THE *DROSOPHILA LATS* GENE ENCODES A PUTATIVE PROTEIN KINASE

We have identified recessive overproliferation mutations by screening and examining clones of mutant cells in genetic mosaics of the fruitfly *Drosophila melanogaster* (Fig. 1A). Flies that carry small groups of somatic cells mutated for negative regulators of cell proliferation or tumor suppressors are viable, yet the overproliferated mutant tissues can be readily identifiable.

One way to generate mosaic animals is to induce mitotic recombination in dev loping heterozygous individuals (Fig. 1B). Recently, it was found that the site-specific r combination system from y ast, the FLP recombinase and its target site FRT, can be used to induce high frequency of mitotic recombination in *Drosophila* (Golic and Lindquist, 1989, Cell 59:499-509; Golic, 1991, Science 252:958-961). To produce and analyze genetic mosaics, a series of special *Drosophila* strains were constructed, containing the FLP/FRT recombination system on genetically marked chromosomes (Xu and Rubin, 1993, Development 117:1223-1237). Using these strains, high frequencies of mosaicism can be produced for more than 95% of the *Drosophila* genes. We have used these strains to identify overproliferation mutations in mosaic animals.

Our results show that screening for overproliferation mutations in mosaic animals is a powerful way to identify negative regulators of cell proliferation and potential tumor suppressor genes. One of the identified genes, large tumor suppressor *(lats)*, has been cloned, and encodes a predicted novel protein kinase. Mutations in *lats* cause dramatic overproliferation phenotypes and various developmental defects in both mosaic animals and homozygous mutants.

6.1. MATERIALS AND METHODS

Genetics

Fly stocks and crosses were grown on standard medium at 25°C unless otherwise indicated. The F1 mosaic screens were modified from the one described in Xu and Rubin (1993, Development 117:1223-1237) and in Xu and Harrison (1994, Methods in Cell Biology 44:655-682). Briefly, the F1 mosaic individuals were produced from three crosses: Mutagenized *y w hsFLP1; P[ry$^+$; hs-neo; FRT]40A* males were mated to the *y w hsFLP1; P[ry$^+$; y$^+$]25F, P[mini-w$^+$; hs-NM]31E, P[ry$^+$; hs-neo; FRT]40A* females. Mutagenized *y w hsFLP1; P[ry$^+$; hs-neo; FRT]42D* males were mated to the *y w hsFLP1;*

P[ry⁺; hs-neo; FRT]42D, P[ry⁺; y⁺]44B, P[mini-w⁺; hs-NM]46F/CyO f mal s. Finally, mutagenized *y w hsFLP1; P[ry⁺; hs-neo; FRT]82B* males were mated to the *y w hsFLP1; P[ry⁺; hs-neo; FRT]82B, P[mini-w⁺; hs-πM]87E, Sb^{63b}, P[ry⁺; y⁺]96E* females. The male parents were irradiated with X-rays (4000 r) and were removed from the crosses after four days of mating. The eggs from the crosses were collected for every 12 hours and aged for another 30 hours before being incubated in a 38°C water bath for 60 minutes. The F_1 animals were then returned to normal culture conditions until eclosion. About 25,000 F_1 adults from these crosses were examined. Each P-induced lethal mutation was recombined onto one of the FRT-carrying arms using the neo^R and w^- double selection as described in Xu and Harrison (1994, Methods in Cell Biology 44:655-682) before examining its clonal phenotype.

The $lats^{x1}$ mutation was meiotically mapped to the right of *claret*. It was further localized to the 100A1-5 region since it complemented *Df(3R)*tll^{g}(100A2-5; 100C2-3) and failed to complement *Df(3R)*tll^{P8x}(100A1-2; 100B4-5) and *Df(3R)*tll^{20}(100A1-3; 100B1-2). A saturation genetic screen had previously been performed for this interval, and three lethal complementation groups, l(3)100Aa, l(3)100Ab and the *zfh-1*, were isolated (Lai et al., 1993, Proc. Natl. Acad. Sci. USA 90:4122-4126). The $lats^{x1}$ mutation failed to complement the EMS-induced mutations in l(3)100Aa ($lats^{a1-a15}$), but complement mutations in l(3)100Ab and *zfh-1*. The clonal phenotypes were examined for $lats^{x1, P1, a1, a2, a6 \text{ and } a10}$ induced either with the FLP/FRT-marker system or X-ray irradiation.

The $lats^{P1}$ allele was recovered from a mosaic male produced from the cross of *y w hsFLP1; P[ry⁺; hs-neo; FRT]82B* x *y w P[lacZ; w⁺]5; P[ry⁺; hs-neo; FRT]82B/delta2-3, Sb*. The mutant chromosome was cleaned up before performing complementation tests and an excision screen (Robertson et al., 1988, Genetics 118:461-470). Two hundred and fifteen excision lines were established that had lost the w^+ gene in the *P[lacZ; w⁺]* element (Bier et al., 1989, Genes Dev.

3:1273-1287). In about 50% of thes lines, the pupal lethality had been reverted completely to wild type, indicating the mutant phenotype is caused by th P-element insertion. Five lin s were found to caus lethality at late embryonic and/or early first instar larval stages. The remaining lines were found to cause lethality at larval and pupal stages or to produce viable mutant animals. All of these mutant excision lines (except one which is located outside the 100A1-5 region) failed to complement *lats*^{x1}and *lats*^{P1}, but do complement mutations in the *zfh-1* and l(3)100Ab loci.

The insert in *lats* cDNA A2 was cloned into the pCaSpeR-hs vector (Thummel and Pirrotta, 1992, Drosophila Inform. Service 71:150) for germ line transformation. Three of the transformed lines were tested and were able to rescu the lethality of the *lats*^{a1}/*lats*^{x1}, *lats*^{P1}and *lats*$^{e26-1}$ animals after one hour heat shock for every 24 hours during larval and pupal development.

Histology

Fixation and sectioning (2 mm) of adult *Drosophila* tissues were performed as described (Tomlinson and Ready, 1987, Dev. Biol. 123:264-275). Scanning electron microscopy was performed as described (Xu and Artavanis-Tsakonas, 1990, Genetics 126:665-677).

Nucleic Acid Manipulation

A P1 genomic clone (DS02640) mapped in the 100A1-7 region was obtained from the Berkeley Drosophila Genome Center (personal communication; Hartl et al., 1994, Proc. Natl. Acad. Sci. USA 91:6824-6829). DNA fragments from this P1 clone and genomic DNA obtained by plasmid rescue from the *lats*^{P1} mutant (Bier et al., 1989, Genes Dev. 3:1273-1287) were used to isolate several overlapping cosmids including CLT-52 from the genomic library prepared by J. Tamkun. Genomic DNA from +7.5 (*Bgl*II) to -4.2 (*Eco*RI; Fig. 3) was used to screen a total imaginal disc cDNA library prepared by A. Cowman.

Screening approximately 2 million phag yielded three groups of cDNAs (five *lats* cDNAs; fifteen T1 cDNAs; fourteen T2 cDNAs). The sizes of th inserts in the *lats* cDNAs are as f llows: 5.6 kb in A2; 5.1 kb in B1; 1.1 kb in 9 and 4; and 0.9 kb in B3.

Genomic DNA from $lats^{x1}$/*TM6B*, $lats^{a1\text{-}15}$/*TM6B*, $lats^{P1}$/*TM6B*, $lats^{e7\text{-}2}$/*TM6B*, $lats^{e78}$/*TM6B*, $lats^{e100}$/*TM6B*, $lats^{e119}$/*TM6B* and $lats^{e148}$/*TM6B* flies was digested with a combination of the *Eco*RI, *Bam*HI, *Bgl*II and *Xho*I restriction enzymes for Southern analysis.

DNA Sequencing

DNA sequence was determined by the dideoxy chain termination method (Sanger et al., 1977, Proc. Natl. Acad. Sci. USA 74:5463-5467) using Tag polymerase (Perkin Elmer) and Sequenase (U.S. Biochemical Corp.). The sequences of *lats* cDNAs were determined from both strands using templates generated from plasmids containing *Eco*RI fragments inserted into the pBlueScriptII vector. Templates generated from DNase 1 deletion subclones were also used. The complete sequences of cDNAs A2 and 9 were determined; partial sequences were determined for cDNAs B1 and 4. Templates of genomic DNA were generated from plasmids containing *Eco*RI fragments and were sequenced on one strand using synthetic oligonucleotide primers. Mutant DNA from the $lats^{a1}$ allele was amplified with PCR reactions using synthetic oligonucleotide primers and cloned in the pBlueScript II vector for sequencing.

6.2. RESULTS

Screening for Overproliferation Mutations in Mosaic Animals

We have screened individuals carrying clones of cells that were homozygous for either X-ray or P-element induced mutations for overproliferation phenotypes. (Fig. 1B; Materials and Methods). Two types of overproliferation ph notypes were sought: a) Clones of mutant cells form d

ov rproliferat d, outgrowth tissues in a non-position-dependent fashion; b) Clones of mutant cells formed normal structur s, but proliferated faster than wild-type cells such that the sizes of the mutant clones were larger than their wt twin-spot clones. Three independent mutations were identified that caused the first type of phenotype (Fig. 2A-2E). A mutation which was allelic to one of the original mutations was later found to cause the second type of phenotype (see below). All three mutations in the first class caused embryonic and/or early larval lethality and they represented single alleles of different loci since they had different chromosome locations. One of them was identified among 215 randomly chosen lethal mutations in which each were caused by a P-element insertion in a different essential gene (Karpen and Spradling, 1992, Genetics 132:737-753; Berkeley *Drosophila* Genome Center, personal communication). In addition to these overproliferation mutations, one P-induced mutation was found to cause both unpatterned outgrowth and duplications of patterned structures in mosaic animals, suggesting that this mutation may not directly affect cell proliferation.

The *lats* Locus Is Defined by a Single Complementation Group of Mutations That Cause Defects Throughout Development

The mutations caused different levels of overproliferation. One mutation (*lats*^{x1}) produced much more dramatic overproliferated clones than the ones produced by the other mutations (Fig. 2A, 2B). The *lats* mutant clones induced in first instar larvae can be as large as 1/5 of the body size. Tumorous outgrowth caused by *lats*^{x1} was found in all the tissues that had been examined including eyes, legs, wings, heads, notums, antenna, and abdominal cuticles. The *lats*^{x1} mutation was genetically mapped in the 100A1-5 region and the locus was further defined by a single complementation group of over fifty alleles including mutations induced by

X-ray, EMS, P-el ment insertion and imprecise excision of the P-el m nt (Table 2; Materials and Methods).

TABLE 2

The alleles of the *lats* locus*

Alleles	Phenotypes of homozygous animals	Phenotypes of mutant clones	Representative alleles	No. of alleles
Strong	Late embryonic and early 1st instar larval lethal	Large outgrowth	*lats*^{x1}, *lats*^{a1}, *lats*^{a4}	14
Medium	Late larval and pupal lethal, normal size of animals	Large outgrowth	*lats*^{P1}, *lats*^{e124}	16
	Pupal lethal, giant animals	Large outgrowth	*lats*$^{e26-1}$	3
Weak	Semi-viable and viable: rough eye outgrowth on head, wing held-out, sterile	Mutant clones larger or normal in size	*lats*^{a10}, *lats*$^{e53-2}$	17

* The various alleles of the *lats* gene are classified into three main groups as indicated in the left column. Their phenotypes, displayed in either homozygous mutant animals or clones of mutant cells in mosaic animals, are listed in the next two columns respectively. For a given viable or semi-viable allele, the homozygous mutant animals display one, two, three, or all four of the listed phenotypes. Representative alleles and the numbers of alleles for each group are given in the two right columns. The origins of these alleles are described in the Material and Methods.

Removing the P-element insertion reverted the lethal chromosome into wild type, indicating the P-element insertion is responsible for the mutant phenotype. Furthermore, five of the imprecise excision lines caused late embryonic and early larval lethality which were stronger than the pupal lethality phenotype caused by the *lats*^{P1} mutation. These five excision lines failed to complement *lats*^{x1}, but complemented the mutations in two other complementation groups (*l(3)100Ab* and *zfh-1*) in the 100A1-5 region, indicating that these two genes were not affected by the excision alleles.

The *lats* alleles can be classified into three main groups (Table 2). Strong allel s caused homozygous animals to die at a late embryonic stage or shortly after hatching

with no obvious cuticular defect. Mutations in the group of medium alleles cause lethality at different times in larval and pupal development. This group was further divided into two subgroups b cause three of the excision alleles not only caused pupal lethality, but the sizes of the homozygous mutant animals were also significantly larger than wt animals (Fig. 2C). The weak mutations caused either one or a combination of the following phenotypes: held out wings with broadened blades, rough eye with ventral outgrowth, outgrowth on the dorsal-anterior region of the head and partial to complete sterility (Table 2).

Proliferation defects were observed in both mutant clones in mosaic animals and homozygous mutants. Clones of cells on the head that were homozygous for strong or medium alleles formed unpatterned, overproliferated tissues with many lobes or folds. The mutant cells seemed to be "budding out" of the surface to form new proliferation centers or lobes (Fig. 2A, 2F, 2H). The sizes and the shapes of these mutant cells were very irregular. Cells several times larger than their neighbors were often seen in mutant clones, indicating problematic cell division (Fig. 2F, 2G). Furthermore, *lats* mutant clones behaved differently from clones mutant for the previously identified *Drosophila* tumor suppressor genes such as *dlg*, *lgl* and *hyd*. The *dlg*, *lgl* or *hyd* mutant cells proliferated slower than wt cells and thus, the mutant clones induced in first instar larvae were competed away during growth and did not form detectable clones in the adults (Bryant, 1987, Experimental and genetic analysis of growth and cell proliferation in *Drosophila* imaginal discs, *in* "Genetic Regulation of Development," A.R. Liss, New York, pp. 339-372; Woods and Bryant, 1989; Dev. Biol. 134:222-235; Mansfield et al., 1994, Dev. Biol. 165:507-526; Allen Shearn, personal communication). In contrast, the *lats* mutant clones induced at similar developmental stages formed dramatic overproliferated tissues, suggesting the mutant cells proliferated faster than wt c lls. Consistent with this notion, clones of cells

mutant for a weak *lats* allele (*lats*^{a10}) produced normal looking tissues, but the mutant clones were significantly larg r than th ir wt twin-spot clones. In hom zygous animals, the imaginal discs and the central nervous system in many of the pupal lethal mutants were dramatically overproliferated (Fig. 2D, 2E). The discs lost the single layer of epithelial structure and formed multi-layer, deformed tissues. The *lats* overproliferation phenotype was not caused by prevention of differentiation. Cells in the overproliferated mutant clones on the body differentiated and produced bristles and hairs, although the morphologies of these structures were not wild type (Fig. 2I-2L). Careful examination of multiple mutant clones confirmed that *lats* caused mutant cells (*w*$^-$ cells in the eye, *y*$^-$ bristles and enlarged-base hairs on the body) to overproliferate and did not affect the surrounding wt tissues. Finally, the frequency of overproliferated clones was similar to wt clonal frequency induced with the same FRT element, indicating that loss of the *lats* function alone is sufficient to initiate the overproliferation process.

Cloning of the *lats* Gene

Genomic DNA from the 100A1-5 region was isolated using probes mapped to this region (Materials and Methods). A restriction map of the relevant genomic region is illustrated in Figure 3. Genomic DNA flanking the P-insertion site (+7.5 to -4.2) was used to screen a total imaginal disc cDNA library. A group of cDNAs corresponding to a 5.7 kb transcript (*lats*) was found to contain sequence from the region where the P-element was inserted (Fig. 3). Two other groups of cDNAs were also isolated (T1 and T2). The 5.7 kb transcript was located in an intron of the T1 gene (Fig. 3). The intron-exon structure of the 5.7 transcription unit was determined by Southern and sequence analysis of the cDNA clones and the corresponding genomic DNA (Materials and Methods). The *zfh-1* gene was found to be located at the left side of the 5.7 kb transcription unit (Fig. 3; Fortini et al., 1991, Mechanisms of Development 34:113-122).

In addition t *lats*^{Pl}, genomic DNA from the five strong excision alleles was analyzed. All of them deleted exon sequenc s from the 5.7 kb transcript and, in addition, three of them also deleted sequences in the next transcript (T2; Fig. 3). Furthermore, DNA from the X-ray and EMS induced mutants was analyzed with cDNA probes made from the 5.7 kb, T2 and T1 transcripts. In two cases alterations were detected in the 5.7 kb transcription unit: a 0.4 kb and a 0.3 kb deletions associated with *lats*^{a1} and *lats*^{a4}, respectively (Fig. 3). The 446 bp deletion in *lats*^{a1} was revealed by sequencing. It removed codons 92 to 238 of the open reading frame and caused a frame shift from codon 239 (Fig. 5). Finally, transformants containing a cDNA corresponding to the 5.7 transcript driving by the hsp70 promoter rescued the lethality of both strong and medium *lats* alleles. These findings indicate that the 5.7 kb transcription unit which correspond to the *lats* gene and strong *lats* alleles including *lats*^{a1} were either amorphic or nearly amorphic alleles.

The *lats* Gene Encodes a Putative <u>Protein-Serine/Threonine Kinase</u>

The 5.7 kb *lats* transcript was detected throughout development (Fig. 4) and in both adult males and females (data not shown). In addition, probes from the 5.7 kb transcript also detected a second transcript, which is about 1 kb shorter (4.7 kb), in young embryos (0-4 hrs; Fig. 4) and in adult males and females. Northern analysis showed there was more maternally deposited 4.7 kb transcripts than 5.7 kb transcripts in young embryos (0-2 hrs; Fig. 4). The 5.7 kb transcript became the dominant message at the embryonic stage (4-6 hrs), known to have zygotic gene expression (Fig. 4). No effort was made to isolate cDNA clones corresponding to the 4.7 kb transcript; thus the exact sequence of this short transcript is not known. However, a polyadenylation signal consensus sequence was found at nucleotide position 4655 - 4660 in the 5.7 kb transcript and in the corresponding genomic DNA (Fig. 5) and a 0.51 kb probe from the 3' end of

the 5.7 kb transcript did not hybridiz to the 4.7 kb transcript while a 1 kb probe from the 5' untranslated region of the 5.7 kb transcript hybridized to both the 5.7 kb and 4.7 kb transcripts. This suggests that the 4.7 kb transcript may be a truncated version of the 5.7 kb transcript. The genomic and cDNA sequence corresponding to the 5.7 kb transcript was determined (Materials and Methods). The entire 5720 bp cDNA sequence, which is interrupted by seven introns, and the putative *lats* product (lats), deduced from the long open reading frame, are illustrated in Figure 5. An interesting feature of the 5.7 kb transcript is the existence of a 141 bp segment located in the 3' untranslated region (Fig. 5), which is identical to the first 141 bp of the 5' untranslated region of the class I transcript from the *Drosophila* phospholipase C gene, *plc-21* (Shortridge et al., 1991, J. Biol. Chem. 266:12474-12480). The functional significance of this sequence motif is unknown. It could be a regulatory target sequence that is shared by both genes.

There are 34 differences between the *lats* cDNA and genomic sequences and 31 of them do not affect the deduced amino acid sequence. In the remaining three differences, one changes the serine 206 in cDNA into a cysteine. The second change in the genomic sequence adds an additional glutamine in the poly-glutamine opa repeat (Fig. 6; Wharton et al., 1985, Cell 40:55-62). The third is the addition of a fifteen bp sequence in the genomic DNA after the nucleotide 2644 of the cDNA. This sequence could be translated into another copy of the Arg-Glu-Arg-Asp-Gln (part of SEQ ID NO:2) peptide. However, this sequence is not present in the two independent cDNA clones that were sequenced.

The predicted lats product contains 1099 amino acid residues. The kinase domain of lats is more similar to protein-serine/threonine kinases than to protein-tyrosine kinases, especially in the sequences of the domains VI and VIII defined by Hanks et al. (1988, Science 241:42-52); protein-serine/threonine kinase cons nsus in domain VI: Asp-Leu-Lys-Pro-Glu-Asn (SEQ ID NO:9). Lats sequence in domain

VI: Arg-Asp-Ile-Lys-Pro-Asp-Asn (836-842) (part of SEQ ID NO:2); protein-serine/threonine kinase consensus in domain VIII: Gly-Thr/Ser-X-X-Tyr/Phe-X-Ala-Pro-Glu (SEQ ID NO:10). Lats sequenc in domain VIII: Gly-Thr-Pro-Asn-Tyr-Ile-Ala-Pro-Glu (917-925) (part of SEQ ID NO:2). The C-terminal half of lats shares extensive sequence similarity with a group of six proteins including the Dbf20 and Dbf2 cell cycle protein-ser/thr kinases from *Saccharomyces cerevisiae* (Johnston et al., 1990, Mol. Cell. Biol. 10:1358-1366; Toyn et al., 1991, Gene 104:63-70; Toyn and Johnston, 1994, EMBO J. 13:1103-1113), and the COT-1 putative protein kinase from *Neurospora crassa* (Yarden et al., 1992; EMBO J. 11:2159-2166) (Fig. 6A, 6B). The sequence similarity between the kinase domains of lats and these proteins (39-49% identity) is much higher than the sequence similarity observed between the different subgroups of protein-ser/thr kinases (20-25% identity; Hanks et al., 1988, Science 241:42-52). However, there is an insertion of about 40 amino acid residues within the kinase domains of these proteins, sharing little sequence similarity (denoted by a black bar in Fig. 6B). The human myotonic dystrophy protein kinases (MDPK) also have significant similarity with the C-terminal region of lats (Brook et al., 1992, Cell 68:799-808; Fu et al., 1993; Science 260:235-238, Mahadevan et al., 1993, Hum. Mol. Genet. 2:299-304), but their kinase domains do not contain this ~40 amino acid insertion. In addition, lats and these proteins also share significant levels of sequence similarity in the two regions (each contains ~100-150 amino acids) flanking the kinase domain (20-28% identity; Fig. 6A, 6B). In the case of Dbf20, its entire sequence except for the 20 C-terminal most residues can be aligned with lats, indicating lats is a close relative of Dbf20. A poly-glutamine opa repeat is located near the middle of the protein (Fig. 5; Wharton et al., 1985, Cell 40:55-62). The N-terminal half of lats contains many short homopolymeric runs including poly-prolin which makes up about 15% of the residues. At least one of the proline-rich stretches closely

match s the consensus of SH3-binding sites (Fig. 3B; Ren et al., 1993, Science 259:1157-1161), raising the possibility that it may interact with SH3-containing proteins. No putativ signal sequ nce app ars in the lats protein, indicating that it is an intracellular protein.

6.3. DISCUSSION

Screening for Mutations in Mosaic Animals to Identify and Study Potential Tumor Suppressors

The comparison between mosaic flies and tumor patients is simplistic yet useful. Tumor patients contain wt tumor suppressor genes in most of their cells and only small groups of cells sustain mutations in tumor suppressors. We have searched for recessive overproliferation mutations in mosaic animals. Flies that carry somatic cells mutated for tumor suppressors or negative regulators of cell proliferation are viable, yet the overproliferation mutant phenotype is readily identifiable. Therefore, mosaic flies, which are in a fashion analogous to tumor patients, provide a mean to screen for potential tumor suppressors. Three overproliferation mutations were identified in our screen. They were not identified as "interesting" mutations in screens for embryonic lethal mutations. Identifying overproliferation mutations in homozygous mutant larvae and pupae is not only biased against embryonic lethals, but also laborious, since it requires establishment of individual lines before examining the potential phenotypes. Further screens for overproliferation mutations in mosaic animals will allow us to identify other important players in pathways that negatively regulate cell proliferation.

The overproliferation phenotypes that we observed were caused by loss of function in a single gene. In humans, it was suggested that most retinoblastomas are caused by defects in a single tumor suppressor (Knudson, 1971, Proc. Natl. Acad. Sci. USA 68:820-823). On the other hand, evidence indicates that tumorigenesis in other human tissues (*e.g.*,

colon cancer) is a multistep process which involves inactivation of more than one gene (Fearon and Vogelstein, 1990, Cell 61:759-767; Vog lstein and Kinzler, 1993, Trends Genet. 9:138-141). Overproliferation caused by defects in multiple genes is unlikely to be detected in our screens unless these genes are located on the same chromosome arm. To identify this type of gene, one could perform a modified mosaic screen which induces clones of cells to become homozygous for more than one mutagenized chromosome arm.

lats Affects Many Tissues Throughout Development

The *lats* gene is genetically defined by a single complementation group that consists of various alleles causing a wide range of defects. Different alleles were found to cause lethality at almost every stage during development: embryo, early larvae, late larvae, early pupae, late pupae and pharate-adult. The embryonic lethality occurs in the pharate first instar stage. The early embryonic requirements for *lats* could well be masked by the wt products that are maternally deposited in the egg. Weak *lats* alleles produce viable animals with phenotypes ranging from rough eye to sterility. The *lats* transcripts were detected throughout development up to adult stage, consistent with the observation that *lats* mutants affect all these stages. Although mutations at *lats* cause many defects, affecting cell proliferation could cause most of the phenotypes including overproliferation in mutant clones, lethality at the various stages, tissue overproliferation on the head, broadened wing blade, and sterility in homozygous mutants. However, phenotypes such as extra cuticle deposits and malformed bristles and hairs are evidence of defects in differentiation.

The different behavior of the *lats* mutant clones and clones mutant for other previously identified *Drosophila* tumor suppressors is interesting. Cells mutant for *dlg*, *lgl* or *hyd* seem to fail to rec ive growth regulation signals. They proliferated slower than wt cells during larval stages

when th cells were instructed to proliferate, and they failed to terminate proliferati n in late larval and pupal stages when th wt cells hav ceas d proliferation. On the other hand, the *lats* mutant clones induced during the larval stages were overproliferated, and later the mutant cells on the body were differentiated to form adult cuticular structures. Thus, *lats* could be a negative regulator that monitors the rate of proliferation.

The *lats* gene is located in a complex region. The 5' end of the *lats* 5.7 kb transcript (cDNA) is only about 550 bp away from the T2 transcript and its 3' end is about 1.5 kb away from the *zfh-1* transcript. Furthermore, all three of these closely located transcripts are located in an intron of the T1 transcription unit. Thus, a sizable deletion in the 5.7 kb transcription unit could affect the function of any of the genes in the region, which makes it difficult to determine which transcript is responsible for the *lats* phenotype. The fact that P-element transform lines carrying a cDNA from the 5.7 kb transcript under the *hsp70* promoter rescued all types of *lats* alleles demonstrated that the 5.7 kb transcription unit is the *lats* gene.

The lats Putative Protein-Ser/Thr Kinase Shares Homology With Proteins That Are Involved in Regulation of Cell Cycle <u>and Growth in Budding Yeast and *Neurospora*</u>

All 11 subdomains of the kinase domain that are found in previously identified protein kinases (Hanks et al., 1988, Science 241:42-52) are conserved in lats. This predicts that lats is a protein kinase. Furthermore, the sequence comparisons suggest lats to be a ser/thr kinase as the lats kinase domain is more similar to protein-ser/thr kinases than to protein-tyr kinases. The C-terminal half of lats shares extensive sequence similarity with a group of six proteins. Mutations are known for three of these genes and in each case they affect either cell cycle or growth. The *cot-1* (colonial temperature sensitive-1) gene of *Neurospora* was identified by a temperature sensitive mutant that causes compact colony

growth (Mitchell and Mitchell, 1954, Proc. Natl. Acad. Sci. USA 40:436-440; Galsworthy, 1966, Diss. Abstr. 26:6348). Wild-type filamentous ascomycete *Neurospora* grows on solid media by continuous hyphal elongation and branching to form spreading colonies. Strains lacking functional *cot-1* gene are viable, but their hyphae branch extensively, resulting in compact colonial growth (Yarden et al., 1992, EMBO J. 11:2159-2166). This extensive branching phenotype is somewhat similar to the growth property of the *lats* mutant clones: the *lats* mutant cells continue to "bud" out of the surface to form new proliferation lobes. Another homologous gene, the *DBF2* gene of the budding yeast, was identified in a genetic screen for mutations causing defects in DNA synthesis (Johnston and Thomas, 1982, Mol. Gen. Genet. 186:439-444). The temperature sensitive alleles of *DBF2* were found to both delay the initiation of S phase and also to arrest the cell cycle during nuclear division (Johnston et al., 1990, Mol. Cell. Biol. 10:1358-1366). The *DBF20* gene was identified through cross hybridization with *DBF2* DNA (Toyn et al., 1991, Gene 104:63-70). Strains carrying deletions for either *DBF2* or *DBF20* are viable; however, deleting both genes in the same strain causes lethality. The kinase activities of both proteins have been shown to be specific for serine/threonine residues and are regulated during the cell cycle (Toyn and Johnston, 1994, EMBO J. 13:1103-1113). In the case of Dbf20, its entire sequence except the 20 most C-terminal residues can be aligned with lats. The mutant phenotype of *lats* and its sequence homology with the cell cycle protein kinases is consistent with the notion that *lats* might be directly involved in regulation of the cell cycle. The N-terminal half of lats contains many proline-rich stretches and at least one of them closely matches the consensus sequence of SH3 binding sites (Ren et al., 1993, Science 259:1157-1161), raising the possibility that this region could be a regulatory domain for the lats kinase, which binds to SH3 domain-containing proteins.

In recent years, many protein kinases have been identified to be involved in regulation of the cell cycle and c ll proliferation. While Wee1 is an inhibitor of the Cdc2 kinase (Russell and Nurse, 1987, Cell 49:559-567; Featherstone and Russell, 1991, Nature 349:808-811), all other previously identified protein kinases are positive regulators of cell proliferation. They are either required for completion of the cell cycle or for signalling cells to proliferate. Lats is the first predicted protein-ser/thr kinase that has been shown to cause overproliferation when its function is removed. Studies of *lats* and other overproliferation mutations in *Drosophila* will provide a better understanding of how cell proliferation is regulated during development and how mutations could lead to abnormal growth.

7. ISOLATION AND CHARACTERIZATION OF MAMMALIAN *LATS* HOMOLOGS

As described herein, we have cloned and sequenced both mouse and human *lats* homologs.

7.1. ISOLATION AND CHARACTERIZATION OF MOUSE *LATS* HOMOLOGS

cDNA clones for two different *lats* homologs in mice were obtained as follows.

Screening of Mouse Homologs:

Probe:	A 2.2 kb *Bam*HI fragment containing the kinase domain of the *Drosophila lats* gene was labeled with ^{32}P by random labeling		
Library:	Newborn mouse brain lambda ZAP cDNA library from Stratagene		
Hybridization Condition:	45°C, overnight in	6x	SSC
		5x	Denhart's
		0.5%	SDS (sodium dodecyl sulfate)
		100 μg/ml	salmon sperm DNA
Wash:	50°C, 30 min. x 4, in	2x	SSC
		0.1%	SDS

Results: Three positive clones were identified. (M41 clone for th *m-lats* gen , and M51 and M31 clones for the *m-lats2* g ne.)

Two different mouse *lats* hom logs, termed *m-lats* and *m-lats2*, respectively, were isolated and sequenced. Both the m-*lats* and *m-lats2* clones are missing a small amount of the 5' end of their respective genes. The cDNA sequence (SEQ ID NO:5) and deduced protein sequence (SEQ ID NO:6) of *m-lats* are shown in Figure 7. The cDNA sequence (SEQ ID NO:7) and deduced protein sequence (SEQ ID NO:8) of *m-lats2* are shown in Figure 8.

Portions of both the *m-lats* and *m-lats2* cDNAs were used as probes to screen a mouse genomic library, under standard hybridization conditions. Genomic clones for both *m-lats* and *m-lats2* have been isolated that contain most of the coding regions of these genes.

7.2. ISOLATION AND CHARACTERIZATION OF HUMAN *LATS* HOMOLOGS

cDNA clones for at least one human *lats* homolog were obtained as follows.

Screening of Human Homologs (moderately stringent conditions):

Probe: A 2.1 kb PstI fragment containing the kinase domain of the *m-lats* gene was labeled with ^{32}P by random labeling

Library: Fetal human brain lambda gt10 cDNA library from Clontech

Hybridization Condition:	55°C, overnight in	6x	SSC
		5x	Denhart's
		0.5%	SDS
		100 μg/ml	salmon sperm DNA
Wash:	60°C, 30 min. x 2, in	1x	SSC
		0.1%	SDS

Results: About 20 positive clones were identified for the *h-lats* gene.

One human *lats* homolog, termed *h-lats*, was isolated and sequenced. The cDNA sequence (SEQ ID NO:3) and deduced

protein s quence (SEQ ID NO:4) of *h-lats* are shown in Figur 9. Th d duced protein sequence is full-length. The c mpl te coding sequence of the *h-lats* cDNA was inserted into a bact rial cloning vector (derived from Bluescript (KS)-vector; Stratagene) to form plasmid pBS(KS)-h-lats (Fig. 10). The total size of pBS(KS)-h-lats is 6.96 kb.

A *h-lats* cDNA fragment was used as a probe under conditions of moderate stringency to screen a human genomic cosmid library. Genomic *h-lats* clones were isolated. Over 70 kb of the genomic *h-lats* sequence has been isolated; the isolated sequences include all of the *h-lats* coding sequence (but not all the exon sequences).

An *m-lats2* cDNA fragment was used as a probe to screen a human genomic phage library under the conditions described above, except that hybridization was carried out at 50°C, and washing was carried out at 55°C with 2X SSC, 0.1% SDS. Two genomic *h-lats* clones have been isolated that specifically hybridize to *m-lats2* cDNA probes and do not hybridize to *m-lats* and *h-lats* cDNA probes.

8. CONSERVATION OF SEQUENCES AND DOMAIN STRUCTURE AMONG LATS HOMOLOGS OF DIFFERENT SPECIES

Comparison of the sequences of *Drosophila* lats, h-lats, m-lats, and m-lats2 showed a startlingly high degree of sequence conservation, both overall and within domains of the lats protein. An alignment of the h-lats (SEQ ID NO:4) and m-lats (SEQ ID NO:6) protein sequences is shown in Figure 11. The overall amino acid sequence identity between h-lats and m-lats is 93%. An alignment of the h-lats (SEQ ID NO:4) and m-lats2 (SEQ ID NO:8) protein sequences is shown in Figure 12.

Homologous domains (*i.e.*, domains conserved) between the different lats homologs were identified. Figure 13 presents an alignment of the h-lats protein sequence (SEQ ID NO: 4) and the *Drosophila* lats protein sequence (SEQ ID NO:2), and indicates the domains identified as conserved among the lats homologs from th various species.

The identified domains were as follows:

(1) Lats C-t rminal domain 3 (LCD3)

The last three amino acids (VYV) are completely conserv d in all four homologs including *Drosophila* lats, h-lats, m-lats, and m-lats2.

(2) Lats C-terminal domain 2 (LCD2)

	amino acid residues
h-lats	1077-1086
Drosophila lats	1075-1084

This domain is completely conserved in all four homologs including *Drosophila* lats, h-lats, m-lats, and m-lats2 (10/10 identical residues).

(3) Lats C-terminal domain 1 (LCD1)

	amino acid residues
h-lats	1032-1043
Drosophila lats	1035-1047

This domain is completely conserved among *Drosophila* lats, h-lats, and m-lats (12/12 identical), and is highly conserved between any of the foregoing and m-lats2 (11/12 identical).

(4) Kinase domain

	amino acid residues
h-lats	703-1014
Drosophila lats	711-1018

This domain is highly conserved among the four homologs (76% identical between *Drosophila* lats and h-lats; 99% identical between h-lats and m-lats; 83% identical between h-lats and m-lats2).

A potential phosphorylation residue in *Drosophila* lats and the mammalian homologs that could lead to the activation of the lats kinases after phosphorylation was identified.

Activities of protein kinases are often regulated by varying the phosphorylation state of specific serine, threonine, and tyrosine residues. Phosphorylation of a serine or threonine within twenty residues upstream of

an Ala-Pro-Glu consensus in subdomain eight of the kinase domain, is often required for catalytic activities of many protein-ser/thr kinases (Hanks t al., 1988, Science 241:42-52). For example, Thr167 and Thr197 are phosphorylated in Cdc2 of fission yeast and in the cardiac muscle adenosine 3',5'-phosphate dependent protein kinase, respectively (Ducommun et al., 1991, EMBO J. 10:3311-3319; Gould et al., 1991, EMBO J. 10:3297-3309; Shoji et al., 1983, Biochem. 22:3702-3709). A ser residue in a similar position of the lats kinase domain is conserved in *Drosophila* lats, h-lats, m-lats, and m-lats2 (Ser914 in *Drosophila* lats; Ser909 in h-lats). Thus, the activities of *Drosophila* lats and its mammalian homologs may be regulated by phosphorylation of this ser residue.

(5) Lats flanking domain (LFD)

	amino acid residues
h-lats	607-702
Drosophila lats	612-710

LFD is a domain that flanks and is amino-terminal to the kinase domain. This domain is highly conserved between *Drosophila* lats and h-lats (68% identical) and is also highly conserved between h-lats and m-lats2 (71% identical). This domain is completely conserved between h-lats and m-lats (100% identical).

(6) Lats split domain 1 (LSD1)

		amino acid residues
LSD1	*Drosophila*-lats	365-392
LSD1 anterior (LSD1a)	h-lats	328-334
LSD1 posterior (LSD1p)	h-lats	498-518

Certain lats domains have been termed split domains because the amino- (anterior) and carboxy- (posterior) portions of the domain appear separated from each other in at least one of the lats homologs. Split domains may constitute discontinuous binding/functional regions (*e.g.*, brought together by tertiary structur). The LSD1a subdomain is completely conserved among *Drosophila*

lats, h-lats, and m-lats (7/7 identical), and is not conserved in m-lats. The LSD1p subdomain is conserved b tween th four homologs (14/21 identical among *Drosophila* lats, h-lats, and m-lats; 13/21 identical between h-lats and m-lats2). The LSD1a and LSD1p subdomains are adjacent to each other in *Drosophila* lats and are separated in the mammalian homologs.

(7) Lats split domain 2 (LSD2)

		amino acid residues
LSD2	*Drosophila* lats	536-544
LSD2 anterior (LSD2a)	h-lats	28-31
LSD2 posterior (LSD2p)	h-lats	555-559

Both the LSD2a and LSD2p subdomains are completely conserved among the four homologs. However, the two subdomains are adjacent to each other in *Drosophila* lats and are separated in the mammalian homologs.

(8) Putative SH3-binding domain (SH3-binding)

	amino acid residues
h-lats	247-268
Drosophila lats	196-217

This domain is highly conserved among *Drosophila* lats, h-lats, and m-lats (10/22 identical), and does not exist in m-lats2.

The opa domain does not appear in the mammalian lats homologs.

9. FUNCTIONAL INTERCHANGEABILITY OF THE HUMAN AND *DROSOPHILA* LATS HOMOLOGS

9.1. OVEREXPRESSION OF HUMAN LATS OR OF *DROSOPHILA* LATS CAUSES A SMALLER, ROUGH EYE IN *DROSOPHILA*

Overexpression of lats and h-lats in the developing *Drosophila* eye was carried out. The *Drosophila lats* cDNA and *h-lats* cDNA were each cloned into the pGMR P-element vector. This vector was constructed by Bruce Hay and Gerald M. Rubin at the University of California at Berkeley, and will direct the expression of a cDNA of interest in the posterior region of the developing third instar larval eye imaging disc of

Drosophila. Ten indep nd nt transformant lines for each of th pGMR-*lats* and pGMR-*h-lats* constructs were generated. The adult ey s of all these lines display d a small-rough eye phenotype (eyes smaller than normal, with irregular, rough appearance). This indicates that both *lats* and *h-lats* genes have the same biological effect when they are overexpressed in the developing *Drosophila* eye.

9.2. HUMAN *H-LATS* GENE CAN REPLACE THE *DROSOPHILA* HOMOLOG TO PREVENT DEATH IN *DROSOPHILA* ANIMALS HAVING MUTANT *DROSOPHILA* LATS

The *Drosophila lats* cDNA was cloned into the pCaSpeR-hs vector (Thummel and Pirrotta, 1992, Drosophila Inform. Service 71:150) for germ line transformation of *Drosophila*. Three of the transformed lines were tested and were able to rescue the lethality of the $lats^{a1}/lats^{x1}$, $lats^{P1}$ and $lats^{e26\text{-}1}$ animals after one hour heat shock for every 24 hours during larval and pupal development. The human *h-lats* cDNA (in a XhoI (blunted)-XbaI fragment) from pBS(SK)-h-lats (Fig. 10) was cloned into the HpaI-XbaI sites of the pCaSpeR-hs vector, to produce plasmid pCaSpeR-hs-h-lats (Fig. 14). Plasmid pCaSpeR-hs-h-lats was used for germ line transformant. Three of the pCaSpeR-hs-h-lats transformant lines were tested and were able to rescue the lethality of the $lats^{P1}$ and $lats^{e26\text{-}1}$ animals under the same conditions used in rescuing experiments for the *Drosophila* gene.

10. HUMAN *LATS* EXPRESSION IS FOUND IN ALL NORMAL TISSUES TESTED AND IS ABSENT IN A LARGE NUMBER OF TUMOR CELL LINES

10.1. HUMAN *LATS* EXPRESSION IN NORMAL TISSUES

The expression of human *lats* RNA was investigated in various adult tissues. A 1.2 kb *Bam*HI fragment of the h-lats cDNA was used as a ^{32}P-labeled probe for Northern analysis. Hybridization was to a nylon membrane containing polyA^{+} RNA from various human fetal and adult tissues, obtained from Clontech. The Northern analysis was carried

out according to the recommended instructions of the manufactur r (Clont ch). The results are shown in Figure 15. *h-lats* was express d in every tissue tested (fetal brain, f tal lung, fetal liver, fetal kidney, adult spleen, adult thymus, adult prostate, adult testis, adult ovary, adult small intestine, adult colon, and adult blood leukocytes). Expression was higher in fetal tissues than in adult tissues.

10.2. HUMAN *LATS* EXPRESSION IN VARIOUS TUMOR CELL LINES

The ^{32}P-labeled *Bam*HI fragment of h-lats was used as a probe for Northern analysis, for hybridization to total RNAs isolated from 42 different human tumor cell lines (obtained from the American Type Culture Collection, Rockville, MD). No h-lats expression was detected in 20 of the tumor lines (48%). The name and tissue origin of the tumor cell lines tested, and the results of the Northern analysis are presented in Table 3.

Table 3

Name of tumor lines	Tumor Origin	Expression detected by Northern analyses YES	NO
5637	Bladder		X
RT4	Bladder	±*	
HT-1376	Bladder		X
HT-1197	Bladder		X
BT-20	Breast	X	
BT-474	Breast	X	
ZR-75-1	Breast		X
ZR-75-30	Breast	X	
BT-549	Breast		X
MDA-MB-453	Breast		X
MDA-MB-435S	Breast		X
HBL-100	Breast		X
LoVo	Colon		X
HT-29	Colon	X	
HCT116	Colon	X	
LS 180	Colon		X
DLD-1	Colon	X	
WiDr	Colon	X	

SW480	Colon	X	
Caco-2	Col n	±	
HEL 92.1.7	Erythroleukemia	X	
MOLT-4	Leuk mia	X	
CEM-CM3	Leukemia	X	
K-562	Leukemia	X	
Jurkat	Leukemia		X
HUT 78	Lymphoma	X	
SK-LU-1	Lung		X
A-427	Lung		X
Calu-1	Lung	X	
NCI-H69	Lung	X	
SK-MEL-3	Melanoma		X
SK-MEL-28	Melanoma		X
SK-MEL-31	Melanoma		X
MIA PaCa-2	Pancreas		X
BxPC-3	Pancreas		X
Hs 700T	Pancreas	X	
Hs 766T	Pancreas	X	
RD	Sarcoma		X
A-204	Sarcoma		X
AN3 CA	Uterine	X	
SK-UT-1	Uterine	X	
HEC-1-A	Uterine	±	

*: weak signal

Thus, 48% of the tumor cell lines tested had no detectable *h-lats* expression, whereas 100% of the normal tissues tested had detectable *h-lats* expression. It should be noted that the 48% figure may be an underestimate of the actual number of tumor cell lines that had decreased lats protein level or activity relative to normal tissue, since while lack of *lats* RNA (*i.e.*, a transcriptional block) allows the conclusion that no lats protein is made, tumor cells that expressed the *lats* RNA may still have had no or low lats protein levels and/or activity due to the possible existence of a translational block or the presence of mutation(s) in an expressed lats protein.

11. DEPOSIT OF MICROORGANISM

Bacteria strain *E. coli* TG2 containing plasmid pBS(KS)-h-lats was deposited on March 24, 1995 with the American Type Culture Collection, 1201 Parklawn Drive, Rockville, Maryland 20852, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedures, and assigned Accession No. 69769.

The present invention is not to be limited in scope by the microorganism deposited or the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

SEQUENCE LISTING

(1) ENERAL INFORMATION:

(i) APPLICANT: Xu, Tian
Tao, Wufan
Wang, Weiyi
Zhang, Sheng
Yu, Wan

(ii) TITLE OF INVENTION: NUCLEOTIDE AND PROTEIN SEQUENCES OF LATS GENES AND METHODS BASED THEREON

(iii) NUMBER OF SEQUENCES: 16

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: To Be Assigned
(B) FILING DATE: On Even Date Herewith
(C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5720 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1103..4402

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATCTAGCACG ACGGCAGCAA CAAAACCACG AATTAATTTT ACTAAATTTA AGCCAAACGC 60

GCATCGGAAA TGCCTGAAAA TGCGATTGAA TGCACGCGAA AAGTGATGGG TTGCGAACGC 120

GA TGAATCA AGTGAAAATA CGTCGGCAAA TATCAGCGAA TTGTCGTCAA AAGGCAAGGA 180

AAAACGGAGA AAAAGAGGAA AAGCAATAAG TGCCGTGTGT GGGAAACGCG AAAAAGGCGA 240

GAACAAAGAG GCGAAAAGCG AGGAAATTGC GTGGAAAACG TGGAAAACGC GAAGAAGCGA 300

AGCTCCAAGT TGGCCGCCAT CGATTCGTGC GTAGGATCAA TTAAGATTCC GAGTGGTCGA 360

GAATCGGCTC AAATCAAATT AAAATCAACT AATATTTTGG TATTCAGATA TTCAAATGGA 420

ATTCATTCAT CGCCTGCGAC TTTTATTCGG ATCTGCCAAC TATTTTTGAA TTTGAATTGT 480

GTGTCTGCGG CTGGCGCAGA ATCTCTGATA AAGCAGAGGA ATAAAATCGG AAGAACAACA 540

AATACAAATA CAAATGAAAT GCGGGGAGCA GTATTTACAT GCCAAATGAA TGCTGGATAG 600

GCGAAAGGGG GGGTTTCTCT TATAATGCAA ATGTGAATGT GAATGCGAAT GCGAATGCGA 660

GTGGAAGAAT TCCCGGCGCG AGTGATAAAT AATCCGACGA CAAACAAAGC AGAAGCCTAC 720

ACCGCGAGAA AGAGCAGCGC AAACACAATT ATCTTTATTG AGAGCAACAA TATCAAGATC 780

GAGATAATAA AGCATCCTAA AACCCGCGCC TTAGTTCGTT TTAGTCTCGC CACGGATATA 840

GATATTCAAA GGCAAAAAGG TGGTGTCGGC ATCGCCAGAC AAACAAGTAA AGCATCTATT 900

TCATACAAAA CAACCAATTA AATAATAATA AAAATAATAA TAATCGTAGA GAGGCAGAGC 960

CAAATCAAAT TCCCGGCCGC CGATGTGCCC CAGTGTGTGT GCGTGTGTGT GTGTGTGTGC 1020

TGTGCTGTGC TGTGCGAGTG TTAGTGTGCG GAGCATTTCT GTGATATGAG TGCTAAATGC 1080

CACAGGGCGA AGCAGCAGCA TC ATG CAT CCA GCG GGC GAA AAA AGG GGC GGT 1132
Met His Pro Ala Gly Glu Lys Arg Gly Gly
1 5 10

CGC CCC AAT GAT AAA TAC ACG GCG GAA GCC CTC GAG AGC ATC AAG CAG 1180
Arg Pro Asn Asp Lys Tyr Thr Ala Glu Ala Leu Glu Ser Ile Lys Gln
15 20 25

GAC CTA ACC CGA TTT GAA GTA CAA AAT AAC CAT AGG AAT AAT CAG AAT 1228
Asp Leu Thr Arg Phe Glu Val Gln Asn Asn His Arg Asn Asn Gln Asn
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Tyr Thr Pro Leu Arg Tyr Thr Ala Thr Asn Gly Arg Asn Asp Ala Leu
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ACT CCT GAC TAT CAC CAC GCC AAG CAG CCG ATG GAG CCG CCA CCC TCC 1324
Thr Pro Asp Tyr His His Ala Lys Gln Pro Met Glu Pro Pro Pro Ser
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GCC TCT CCT GCT CCG GAC GTG GTC ATA CCG CCG CCG CCC GCC ATT GTA 1372
Ala Ser Pro Ala Pro Asp Val Val Ile Pro Pro Pro Pro Ala Ile Val
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GGT CAG CCC GGA GCC GGC TCC ATA TCC GTA TCC GGT GTG GGC GTT GGA 1420
Gly Gln Pro Gly Ala Gly Ser Ile Ser Val Ser Gly Val Gly Val Gly
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GTG GTG GGT GTG GCG AAC GGA CGT GTG CCA AAG ATG ATG ACG GCC CTA 1468
Val Val Gly Val Ala Asn Gly Arg Val Pro Lys Met Met Thr Ala Leu
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Ser Ser His Tyr Leu Arg Cys Ser Pro Ala Leu Asp S r Gly Ala Gly
140 145 150

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Ser Ser Arg Ser Asp Ser Pro His Ser His His Thr His Gln Pro Ser
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TCG AGG ACG GTG GGT AAT CCA GGT GGA AAT GGT GGA TTT TCT CCG TCG 1660
Ser Arg Thr Val Gly Asn Pro Gly Gly Asn Gly Gly Phe Ser Pro Ser
175 180 185

CCA AGC GGT TTC AGT GAG GTG GCT CCA CCG GCG CCG CCG CCA CGC AAT 1708
Pro Ser Gly Phe Ser Glu Val Ala Pro Pro Ala Pro Pro Pro Arg Asn
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CCC ACC GCC TCC AGC GCG GCC ACG CCC CCA CCG CCA GTG CCG CCC ACC 1756
Pro Thr Ala Ser Ser Ala Ala Thr Pro Pro Pro Pro Val Pro Pro Thr
205 210 215

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Ser Gln Ala Tyr Val Lys Arg Arg Ser Pro Ala Leu Asn Asn Arg Pro
220 225 230

CCG GCG ATA GCG CCA CCC ACT CAG CGA GGC AAC TCA CCT GTA ATA ACC 1852
Pro Ala Ile Ala Pro Pro Thr Gln Arg Gly Asn Ser Pro Val Ile Thr
235 240 245 250

CAA AAC GGG CTG AAG AAC CCG CAG CAG CAG TTG ACG CAG CAG CTG AAG 1900
Gln Asn Gly Leu Lys Asn Pro Gln Gln Gln Leu Thr Gln Gln Leu Lys
255 260 265

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Ser Leu Asn Leu Tyr Pro Gly Gly Gly Ser Gly Ala Val Val Glu Pro
270 275 280

CCG CCG CCC TAC CTA ATT CAA GGC GGA GCC GGA GGA GCA GCA CCG CCG 1996
Pro Pro Pro Tyr Leu Ile Gln Gly Gly Ala Gly Gly Ala Ala Pro Pro
285 290 295

CCG CCA CCA CCC AGT TAC ACG GCC TCC ATG CAG TCG CGG CAG TCG CCC 2044
Pro Pro Pro Pro Ser Tyr Thr Ala Ser Met Gln Ser Arg Gln Ser Pro
300 305 310

ACA CAA TCC CAA CAA TCG GAC TAC AGG AAA TCC CCG AGC AGT GGG ATA 2092
Thr Gln Ser Gln Gln Ser Asp Tyr Arg Lys Ser Pro Ser Ser Gly Ile
315 320 325 330

TAC TCG GCC ACC TCG GCG GGC TCG CCG AGC CCC ATA ACT GTG TCG CTG 2140
Tyr Ser Ala Thr Ser Ala Gly Ser Pro Ser Pro Ile Thr Val Ser Leu
335 340 345

CCG CCG GCG CCG CTG GCG AAG CCA CAA CCA CGA GTC TAC CAG GCC AGG 2188
Pro Pro Ala Pro Leu Ala Lys Pro Gln Pro Arg Val Tyr Gln Ala Arg
350 355 360

AGT CAG CAG CCG ATC ATC ATG CAG AGT GTG AAG AGC ACG CAG GTC CAA 2236
Ser Gln Gln Pro Ile Ile Met Gln Ser Val Lys Ser Thr Gln Val Gln
365 370 375

AAG CCC GTG CTG CAA ACA GCA GTG GCG CGC CAA TCG CCA TCG AGT GCC 2284
Lys Pro Val Leu Gln Thr Ala Val Ala Arg Gln Ser Pro Ser Ser Ala
380 385 390

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TCG GCC AGC AAT TCA CCA GTC CAC GTG CTG GCC GCT CCA CCC TCT TAC        2332
Ser Ala Ser Asn Ser Pr  Val His Val Leu Ala Ala Pro Pro Ser Tyr
395                 400                 405                 410

CCT CAG AAG TCC  CG GCA GTG GTG CAG CAG CAG CAA CAG GCA GCA  CG        2380
Pro Gln Lys Ser Ala Ala Val Val Gln Gln Gln Gln Gln Ala Ala Ala
                415                 420                 425

GCG GCC CAC CAG CAG CAG CAT CAG CAC CAG CAA TCC AAA CCA CCA ACG        2428
Ala Ala His Gln Gln Gln His Gln His Gln Gln Ser Lys Pro Pro Thr
            430                 435                 440

CCA ACC ACA CCG CCC TTG GTG GGT CTG AAC AGC AAG CCC AAT TGC CTG        2476
Pro Thr Thr Pro Pro Leu Val Gly Leu Asn Ser Lys Pro Asn Cys Leu
        445                 450                 455

GAG CCA CCG TCC TAT GCC AAG AGC ATG CAG GCC AAG GCG GCC ACG GTG        2524
Glu Pro Pro Ser Tyr Ala Lys Ser Met Gln Ala Lys Ala Ala Thr Val
    460                 465                 470

GTA CAG CAG CAG CAA CAG CAG CAG CAA CAA CAG CAG GTC CAG CAG CAG        2572
Val Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Val Gln Gln Gln
475                 480                 485                 490

CAG GTG CAA CAG CAG CAG CAA CAG CAG CAA CAG CAA CTG CAG GCC TTG        2620
Gln Val Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Leu Gln Ala Leu
                495                 500                 505

AGG GTG CTC CAG GCA CAG GCT CAG AGG GAG CGG GAT CAA CGG GAG CGG        2668
Arg Val Leu Gln Ala Gln Ala Gln Arg Glu Arg Asp Gln Arg Glu Arg
            510                 515                 520

GAA CGG GAT CAG CAG AAG CTG GCC AAC GGA AAT CCT GGC CGG CAG ATG        2716
Glu Arg Asp Gln Gln Lys Leu Ala Asn Gly Asn Pro Gly Arg Gln Met
        525                 530                 535

CTT CCG CCG CCG CCC TAT CAG AGC AAC AAC AAC AAC AAC AGC GAG ATC        2764
Leu Pro Pro Pro Pro Tyr Gln Ser Asn Asn Asn Asn Asn Ser Glu Ile
    540                 545                 550

AAA CCG CCG AGC TGC AAC AAC AAC AAC ATA CAG ATA AGC AAC AGC AAC        2812
Lys Pro Pro Ser Cys Asn Asn Asn Asn Ile Gln Ile Ser Asn Ser Asn
555                 560                 565                 570

CTG GCG ACG ACA CCA CCC ATT CCG CCT GCC AAA TAC AAT AAC AAC TCC        2860
Leu Ala Thr Thr Pro Pro Ile Pro Pro Ala Lys Tyr Asn Asn Asn Ser
                575                 580                 585

TCC AAC ACG GGC GCG AAT AGC TCG GGC GGC AGC AAC GGA TCC ACC GGC        2908
Ser Asn Thr Gly Ala Asn Ser Ser Gly Gly Ser Asn Gly Ser Thr Gly
            590                 595                 600

ACC ACC GCC TCC TCG TCG ACC AGC TGC AAG AAG ATC AAG CAC GCC TCG        2956
Thr Thr Ala Ser Ser Ser Thr Ser Cys Lys Lys Ile Lys His Ala Ser
        605                 610                 615

CCC ATC CCG GAG CGC AAG AAG ATC TCC AAG GAG AAG GAG GAG GAG CGC        3004
Pro Ile Pro Glu Arg Lys Lys Ile Ser Lys Glu Lys Glu Glu Glu Arg
    620                 625                 630

AAG GAG TTC CGC ATC AGG CAG TAC TCG CCG CAA GCC TTC AAG TTC TTC        3052
Lys Glu Phe Arg Ile Arg Gln Tyr Ser Pro Gln Ala Phe Lys Phe Phe
635                 640                 645                 650

ATG GAG CAG CAC ATA GAG AAC GTG ATC AAG TCG TAT CGC CAG CGC ACG        3100
Met Glu Gln His Ile Glu Asn Val Ile Lys Ser Tyr Arg Gln Arg Thr
                655                 660                 665
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TAT CGC AAG AAT CAG CTG GAG AAG GAG ATG CAC AAA GTG GGA CTG CCC 3148
Tyr Arg Lys Asn Gln Leu Glu Lys Glu Met His Lys Val Gly Leu Pro
670 675 680

GAT CAG ACC CAA ATC GAG ATG AGG AAA ATG CTG AAC CAA AAG GAG AGC 3196
Asp Gln Thr Gln Ile Glu Met Arg Lys Met Leu Asn Gln Lys Glu Ser
685 690 695

AAC TAC ATT CGA TTG AAG CGC GCC AAG ATG GAC AAG AGC ATG TTC GTC 3244
Asn Tyr Ile Arg Leu Lys Arg Ala Lys Met Asp Lys Ser Met Phe Val
700 705 710

AAA CTG AAG CCC ATT GGA GTG GGT GCA TTT GGC GAG GTA ACG CTG GTG 3292
Lys Leu Lys Pro Ile Gly Val Gly Ala Phe Gly Glu Val Thr Leu Val
715 720 725 730

AGC AAA ATC GAT ACC TCG AAC CAT TTG TAT GCG ATG AAA ACC CTG CGG 3340
Ser Lys Ile Asp Thr Ser Asn His Leu Tyr Ala Met Lys Thr Leu Arg
735 740 745

AAA GCG GAC GTT CTC AAG CGG AAT CAG GTG GCA CAC GTG AAG GCC GAG 3388
Lys Ala Asp Val Leu Lys Arg Asn Gln Val Ala His Val Lys Ala Glu
750 755 760

AGG GAT ATC CTC GCG GAA GCC GAC AAT AAC TGG GTG GTG AAG TTG TAC 3436
Arg Asp Ile Leu Ala Glu Ala Asp Asn Asn Trp Val Val Lys Leu Tyr
765 770 775

TAC AGC TTC CAG GAC AAG GAT AAT CTG TAC TTT GTG ATG GAC TAC ATA 3484
Tyr Ser Phe Gln Asp Lys Asp Asn Leu Tyr Phe Val Met Asp Tyr Ile
780 785 790

CCA GGT GGT GAT CTG ATG TCG CTG CTC ATC AAA CTG GGC ATT TTC GAG 3532
Pro Gly Gly Asp Leu Met Ser Leu Leu Ile Lys Leu Gly Ile Phe Glu
795 800 805 810

GAG GAA CTG GCC AGA TTC TAC ATC GCC GAG GTC ACC TGC GCC GTG GAC 3580
Glu Glu Leu Ala Arg Phe Tyr Ile Ala Glu Val Thr Cys Ala Val Asp
815 820 825

AGC GTT CAC AAA ATG GGC TTC ATT CAC AGA GAC ATC AAG CCT GAC AAC 3628
Ser Val His Lys Met Gly Phe Ile His Arg Asp Ile Lys Pro Asp Asn
830 835 840

ATA CTC ATC GAT AGG GAC GGA CAC ATA AAG CTC ACC GAC TTT GGC CTG 3676
Ile Leu Ile Asp Arg Asp Gly His Ile Lys Leu Thr Asp Phe Gly Leu
845 850 855

TGC ACG GGA TTC CGA TGG ACG CAC AAC TCG AAG TAC TAC CAG GAG AAC 3724
Cys Thr Gly Phe Arg Trp Thr His Asn Ser Lys Tyr Tyr Gln Glu Asn
860 865 870

GGC AAT CAC TCG CGC CAG GAC TCG ATG GAG CCC TGG GAG GAA TAC TCC 3772
Gly Asn His Ser Arg Gln Asp Ser Met Glu Pro Trp Glu Glu Tyr Ser
875 880 885 890

GAG AAC GGA CCG AAG CCC ACC GTG CTG GAG AGG CGA CGG ATG CGC GAT 3820
Glu Asn Gly Pro Lys Pro Thr Val Leu Glu Arg Arg Arg Met Arg Asp
895 900 905

CAC CAA AGA GTC CTG GCC CAC TCG CTG GTG GGC ACC CCG AAC TAC ATA 3868
His Gln Arg Val Leu Ala His Ser Leu Val Gly Thr Pro Asn Tyr Ile
910 915 920

GCT CCC GAG GTG CTG GAG AGG AGT GGG TAC ACG CAG CTG TGC GAC TAC 3916
Ala Pro Glu Val Leu Glu Arg Ser Gly Tyr Thr Gln Leu Cys Asp Tyr
925 930 935

TGG AGC GTG GGC GTC ATC CTT TAC GAG ATG CTG GTG GGT CAG CCG CCC 3964
Trp Ser Val Gly Val Ile Leu Tyr Glu Met Leu Val Gly Gln Pro Pro
940 945 950

TTT CTG GCC AAC AGT CCG CTG GAA ACG CAA CAA AAG GTC ATC AAC TGG 4012
Phe Leu Ala Asn Ser Pr Leu lu Thr Gln Gln Lys Val Ile Asn Trp
955 960 965 970

GAG AAA ACG CTG CAT ATT CCG CCG CAG GCC GAG TTA TCC CGC GAG GCT 4060
Glu Lys Thr Leu His Ile Pro Pro Gln Ala Glu Leu Ser Arg Glu Ala
975 980 985

ACG GAC TTG ATA AGG AGG CTC TGT GCG TCG GCT GAC AAG CGG CTG GGC 4108
Thr Asp Leu Ile Arg Arg Leu Cys Ala Ser Ala Asp Lys Arg Leu Gly
990 995 1000

AAG AGC GTG GAC GAG GTC AAG AGC CAC GAC TTC TTC AAG GGC ATC GAC 4156
Lys Ser Val Asp Glu Val Lys Ser His Asp Phe Phe Lys Gly Ile Asp
1005 1010 1015

TTT GCG GAC ATG CGG AAG CAG AAA GCG CCC TAC ATA CCG GAA ATC AAG 4204
Phe Ala Asp Met Arg Lys Gln Lys Ala Pro Tyr Ile Pro Glu Ile Lys
1020 1025 1030

CAC CCA ACG GAC ACA TCC AAC TTT GAT CCC GTG GAT CCG GAG AAG CTG 4252
His Pro Thr Asp Thr Ser Asn Phe Asp Pro Val Asp Pro Glu Lys Leu
1035 1040 1045 1050

CGC TCG AAT GAC TCC ACC ATG AGC AGC GGC GAT GAT GTC GAC CAG AAT 4300
Arg Ser Asn Asp Ser Thr Met Ser Ser Gly Asp Asp Val Asp Gln Asn
1055 1060 1065

GAC CGC ACT TTC CAC GGC TTT TTC GAA TTT ACC TTC CGT CGC TTC TTC 4348
Asp Arg Thr Phe His Gly Phe Phe Glu Phe Thr Phe Arg Arg Phe Phe
1070 1075 1080

GAC GAC AAG CAG CCG CCG GAT ATG ACG GAC GAT CAG GCG CCG GTT TAC 4396
Asp Asp Lys Gln Pro Pro Asp Met Thr Asp Asp Gln Ala Pro Val Tyr
1085 1090 1095

GTC TGA AATGGATGCT CTCCATGTGC CCAACACCAA CACCCCGCCC CCGAATCATT 4452
Val *
1100

GTTAGTCAAA TAGTCACAAA AAGGGGATAG AAACCATTGA GTGGGCTTGC ATTGTAAAGG 4512

AAGCGTGGCT ATAGAATGAA ACTATCTATA TACATTATAT AAATTATAGG AGACAGTAGA 4572

GGCGGGAGCT ACGTATATAC ATACAAATAA TATACATATA TTTGATATAT ATATATATAT 4632

ATATGCCGTA GGGCATGAAC TGAATAAATA TAAAACGGAG CCGAGTAGAG ATGAAACGAG 4692

AGGAGCGAGT CAGGACCTTC GACCTTTAAC TGAACATAGT ATATCCTTGT GCACTACTAC 4752

TCCACAACAA ATATATATTT TTAAATTGTT AGAATTCAAA AGGGACCAAC TGGAAATCGA 4812

ACCTTTCTGG TGCTCAAAGC AAAGCAAAGC AAAGCAAAAC AAAACGCCTT AAACTAAATG 4872

AGACGCGAAT TTACCCAACC ACTTCACTCC TCTCCTTTCT CCACCTCCGA TCGGTGGCCG 4932

GATTCGAACT CAGCAGGCTG GTTGCATCCG GCCATCCCAT TGACTTCCCA TTCAGAATTG 4992

AGATTGCGAG GTGTGCGATG GAGAACGAAC GGAGACCAAA AGTCGCACGG CAGCGATATA 5052

AGCGGGTCTT ATAAGCCTAA TCTAAATCTA AACTGGGAGA ACAGGACCTA TGTATGTCCT 5112

GCTATCCAAT TCGTCTATCA CTGCTCTTCA TCTGTGTACG ACCCCCACCC CCCCCCTCCC 5172

CATCCAAAAG AACAAACTTA GACGTAGCCT ATGTGAAAAG CTAGCAATGT TAGACCAACT 5232

TGTTGAATGC CAAATGAAAT TGTTTAGCCC CACGAGGAAA ACGCGGGGGA AATTCAACAC 5292

TTATTCTCTG ATAGCAAACG GAAAAGAAAG AAAGAAAAAA AAAAACAGAA ACAGTACGAG 5352

AAAATTGTAA TCTTCTTAAT GTAATATTGT AAAGAACACG TTAATTGTAA TCTATGCTAG 5412

AGTTGTGTAG CGCCCTAAGA TGTTTTTTAG TTTATAGACC GCTAACCGTA ATCTAGTTTA 5472

ATTCCTAACA CTAAGCGAGA GTACAGTACA TTGGTTTTTT TGTTTGTCGT AGGTTCGTTG 5532

GAAAATGCTT AACGGGAAAC GATTTGTTTT TCTCTTTAAT TAGCTTCAGT TTGTATGTGC 5592

GTGTGTTTTT ATTATGACTT ATATATAGTC CATCTGAATA TTCGTGGATG GAGCCTATTT 5652

TAAATGTGAG ATCGAGCTAA TTGAAGGAAA TACAAACAAA CTCTGTGTGC CTTGGCCAAT 5712

TAGTTTAC 5720

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1100 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met His Pro Ala Gly Glu Lys Arg Gly Gly Arg Pro Asn Asp Lys Tyr
1 5 10 15

Thr Ala Glu Ala Leu Glu Ser Ile Lys Gln Asp Leu Thr Arg Phe Glu
20 25 30

Val Gln Asn Asn His Arg Asn Asn Gln Asn Tyr Thr Pro Leu Arg Tyr
35 40 45

Thr Ala Thr Asn Gly Arg Asn Asp Ala Leu Thr Pro Asp Tyr His His
50 55 60

Ala Lys Gln Pro Met Glu Pro Pro Pro Ser Ala Ser Pro Ala Pro Asp
65 70 75 80

Val Val Ile Pro Pro Pro Pro Ala Ile Val Gly Gln Pro Gly Ala Gly
85 90 95

Ser Ile Ser Val Ser Gly Val Gly Val Gly Val Val Gly Val Ala Asn
100 105 110

Gly Arg Val Pro Lys Met Met Thr Ala Leu Met Pro Asn Lys Leu Ile
115 120 125

Arg Lys Pro Ser Ile Glu Arg Asp Thr Ala Ser Ser His Tyr Leu Arg
130 135 140

Cys Ser Pro Ala Leu Asp Ser Gly Ala Gly Ser Ser Arg Ser Asp Ser
145 150 155 160

Pro His Ser His His Thr His Gln Pro Ser Ser Arg Thr Val Gly Asn
165 170 175

Pro Gly Gly Asn Gly Gly Phe Ser Pro Ser Pro Ser Gly Phe Ser Glu
180 185 190

Val Ala Pro Pro Ala Pro Pro Pro Arg Asn Pro Thr Ala Ser Ser Ala
195 200 205

Ala Thr Pro Pro Pro Pro Val Pro Pro Thr Ser Gln Ala Tyr Val Lys
210 215 220

Arg Arg Ser Pro Ala Leu Asn Asn Arg Pro Pro Ala Ile Ala Pro Pro
225 230 235 240

Thr Gln Arg Gly Asn Ser Pro Val Ile Thr Gln Asn Gly Leu Lys Asn
245 250 255

Pro Gln Gln Gln Leu Thr Gln Gln Leu Lys Ser Leu Asn Leu Tyr Pro
260 265 270

Gly Gly Gly Ser Gly Ala Val Val Glu Pro Pro Pro Pro Tyr Leu Ile
275 280 285

Gln Gly Gly Ala Gly Gly Ala Ala Pro Pro Pro Pro Pro Pro Ser Tyr
290 295 300

Thr Ala Ser Met Gln Ser Arg Gln Ser Pro Thr Gln Ser Gln Gln Ser
305 310 315 320

Asp Tyr Arg Lys Ser Pro Ser Ser Gly Ile Tyr Ser Ala Thr Ser Ala
325 330 335

Gly Ser Pro Ser Pro Ile Thr Val Ser Leu Pro Pro Ala Pro Leu Ala
340 345 350

Lys Pro Gln Pro Arg Val Tyr Gln Ala Arg Ser Gln Gln Pro Ile Ile
355 360 365

Met Gln Ser Val Lys Ser Thr Gln Val Gln Lys Pro Val Leu Gln Thr
370 375 380

Ala Val Ala Arg Gln Ser Pro Ser Ser Ala Ser Ala Ser Asn Ser Pro
385 390 395 400

Val His Val Leu Ala Ala Pro Pro Ser Tyr Pro Gln Lys Ser Ala Ala
405 410 415

Val Val Gln Gln Gln Gln Gln Ala Ala Ala Ala Ala His Gln Gln Gln
420 425 430

His Gln His Gln Gln Ser Lys Pro Pro Thr Pro Thr Thr Pro Pro Leu
435 440 445

Val Gly Leu Asn Ser Lys Pro Asn Cys Leu Glu Pro Pro Ser Tyr Ala
450 455 460

Lys Ser Met Gln Ala Lys Ala Ala Thr Val Val Gln Gln Gln Gln Gln
465 470 475 480

Gln Gln Gln Gln Gln Gln Val Gln Gln Gln Gln Val Gln Gln Gln Gln
485 490 495

Gln Gln Gln Gln Gln Gln Leu Gln Ala Leu Arg Val Leu Gln Ala Gln
500 505 510

Ala Gln Arg Glu Arg Asp Gln Arg Glu Arg Glu Arg Asp Gln Gln Lys
515 520 525

Leu Ala Asn Gly Asn Pro Gly Arg Gln Met Leu Pro Pro Pro Pro Tyr
530 535 540

Gln Ser Asn Asn Asn Asn Asn Ser Glu Ile Lys Pro Pr Ser Cys Asn

545 550 555 560

Asn Asn Asn Ile Gln Ile Ser Asn S r Asn Leu Ala Thr Thr Pro Pro
565 570 575

Ile Pro Pro Ala Lys Tyr Asn Asn Asn Ser S r Asn Thr Gly Ala Asn
580 585 590

Ser Ser Gly Gly Ser Asn Gly Ser Thr Gly Thr Thr Ala Ser Ser Ser
595 600 605

Thr Ser Cys Lys Lys Ile Lys His Ala Ser Pro Ile Pro Glu Arg Lys
610 615 620

Lys Ile Ser Lys Glu Lys Glu Glu Glu Arg Lys Glu Phe Arg Ile Arg
625 630 635 640

Gln Tyr Ser Pro Gln Ala Phe Lys Phe Phe Met Glu Gln His Ile Glu
645 650 655

Asn Val Ile Lys Ser Tyr Arg Gln Arg Thr Tyr Arg Lys Asn Gln Leu
660 665 670

Glu Lys Glu Met His Lys Val Gly Leu Pro Asp Gln Thr Gln Ile Glu
675 680 685

Met Arg Lys Met Leu Asn Gln Lys Glu Ser Asn Tyr Ile Arg Leu Lys
690 695 700

Arg Ala Lys Met Asp Lys Ser Met Phe Val Lys Leu Lys Pro Ile Gly
705 710 715 720

Val Gly Ala Phe Gly Glu Val Thr Leu Val Ser Lys Ile Asp Thr Ser
725 730 735

Asn His Leu Tyr Ala Met Lys Thr Leu Arg Lys Ala Asp Val Leu Lys
740 745 750

Arg Asn Gln Val Ala His Val Lys Ala Glu Arg Asp Ile Leu Ala Glu
755 760 765

Ala Asp Asn Asn Trp Val Val Lys Leu Tyr Tyr Ser Phe Gln Asp Lys
770 775 780

Asp Asn Leu Tyr Phe Val Met Asp Tyr Ile Pro Gly Gly Asp Leu Met
785 790 795 800

Ser Leu Leu Ile Lys Leu Gly Ile Phe Glu Glu Glu Leu Ala Arg Phe
805 810 815

Tyr Ile Ala Glu Val Thr Cys Ala Val Asp Ser Val His Lys Met Gly
820 825 830

Phe Ile His Arg Asp Ile Lys Pro Asp Asn Ile Leu Ile Asp Arg Asp
835 840 845

Gly His Ile Lys Leu Thr Asp Phe Gly Leu Cys Thr Gly Phe Arg Trp
850 855 860

Thr His Asn Ser Lys Tyr Tyr Gln Glu Asn Gly Asn His Ser Arg Gln
865 870 875 880

Asp Ser Met Glu Pro Trp Glu Glu Tyr Ser Glu Asn Gly Pro Lys Pro
885 890 895

Thr Val L u Glu Arg Arg Arg Met Arg Asp His Gln Arg Val Leu Ala
900 905 910

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His Ser Leu Val Gly Thr Pro Asn Tyr Ile Ala Pro Glu Val Leu Glu
        915                 920                 925

Arg Ser Gly Tyr Thr Gln Leu Cys Asp Tyr Trp Ser Val Gly Val Ile
    930                 935                 940

Leu Tyr Glu Met Leu Val Gly Gln Pro Pro Ph  Leu Ala Asn Ser Pro
945                 950                 955                 960

Leu Glu Thr Gln Gln Lys Val Ile Asn Trp Glu Lys Thr Leu His Ile
                965                 970                 975

Pro Pro Gln Ala Glu Leu Ser Arg Glu Ala Thr Asp Leu Ile Arg Arg
            980                 985                 990

Leu Cys Ala Ser Ala Asp Lys Arg Leu Gly Lys Ser Val Asp Glu Val
        995                 1000                1005

Lys Ser His Asp Phe Phe Lys Gly Ile Asp Phe Ala Asp Met Arg Lys
    1010                1015                1020

Gln Lys Ala Pro Tyr Ile Pro Glu Ile Lys His Pro Thr Asp Thr Ser
1025                1030                1035                1040

Asn Phe Asp Pro Val Asp Pro Glu Lys Leu Arg Ser Asn Asp Ser Thr
                1045                1050                1055

Met Ser Ser Gly Asp Asp Val Asp Gln Asn Asp Arg Thr Phe His Gly
            1060                1065                1070

Phe Phe Glu Phe Thr Phe Arg Arg Phe Phe Asp Asp Lys Gln Pro Pro
        1075                1080                1085

Asp Met Thr Asp Asp Gln Ala Pro Val Tyr Val  *
    1090                1095                1100
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3984 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 231..3623

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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ACCTTTGGGT TGCTGGGACG GACTCTGGCC GCCTCAGCGT CCGCCCTCAG GCCCGTGGCC      60

GCTGTCCAGG AGCTCTGCTC TCCCCTCCAG AGTTAATTAT TTATATTGTA AAGAATTTTA     120

ACAGTCCTGG GGACTTCCTT GAAGGATCAT TTTCACTTTT GCTCAGAAGA AAGCTCTGGA     180

TCTATCAAAT AAAGAAGTCC TTCGTGTGGG CTACATATAT AGATGTTTTC ATG AAG        236
                                                      Met Lys
                                                        1

AGG AGT GAA AAG CCA GAA GGA TAT AGA CAA ATG AGG CCT AAG ACC TTT       284
Arg Ser Glu Lys Pro Glu Gly Tyr Arg Gln Met Arg Pro Lys Thr Phe
        5                   10                  15
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CCT GCC AGT AAC TAT ACT GTC AGT AGC CGG CAA ATG TTA CAA GAA ATT      332
Pro Ala Ser Asn Tyr Thr Val Ser S r Arg Gln M t Leu Gln Glu Ile
     20                  25                  30

CGG GAA TCC CTT AGG AAT TTA TCT AAA CCA TCT GAT GCT GCT AAG GCT      380
Arg Glu Ser Leu Arg Asn Leu Ser Lys Pro Ser Asp Ala Ala Lys Ala
 35                  40                  45                  50

GAG CAT AAC ATG AGT AAA ATG TCA ACC GAA GAT CCT CGA CAA GTC AGA      428
Glu His Asn Met Ser Lys Met Ser Thr Glu Asp Pro Arg Gln Val Arg
                 55                  60                  65

AAT CCA CCC AAA TTT GGG ACG CAT CAT AAA GCC TTG CAG GAA ATT CGA      476
Asn Pro Pro Lys Phe Gly Thr His His Lys Ala Leu Gln Glu Ile Arg
             70                  75                  80

AAC TCT CTG CTT CCA TTT GCA AAT GAA ACA AAT TCT TCT CGG AGT ACT      524
Asn Ser Leu Leu Pro Phe Ala Asn Glu Thr Asn Ser Ser Arg Ser Thr
         85                  90                  95

TCA GAA GTT AAT CCA CAA ATG CTT CAA GAC TTG CAA GCT GCT GGA TTT      572
Ser Glu Val Asn Pro Gln Met Leu Gln Asp Leu Gln Ala Ala Gly Phe
    100                 105                 110

GAT GAG GAT ATG GTT ATA CAA GCT CTT CAG AAA ACT AAC AAC AGA AGT      620
Asp Glu Asp Met Val Ile Gln Ala Leu Gln Lys Thr Asn Asn Arg Ser
115                 120                 125                 130

ATA GAA GCA GCA ATT GAA TTC ATT AGT AAA ATG AGT TAC CAA GAT CCT      668
Ile Glu Ala Ala Ile Glu Phe Ile Ser Lys Met Ser Tyr Gln Asp Pro
                135                 140                 145

CGA CGA GAG CAG ATG GCT GCA GCA GCT GCC AGA CCT ATT AAT GCC AGC      716
Arg Arg Glu Gln Met Ala Ala Ala Ala Ala Arg Pro Ile Asn Ala Ser
            150                 155                 160

ATG AAA CCA GGG AAT GTG CAG CAA TCA GTT AAC CGC AAA CAG AGC TGG      764
Met Lys Pro Gly Asn Val Gln Gln Ser Val Asn Arg Lys Gln Ser Trp
        165                 170                 175

AAA GGT TCT AAA GAA TCC TTA GTT CCT CAG AGG CAT GGC CCG CCA CTA      812
Lys Gly Ser Lys Glu Ser Leu Val Pro Gln Arg His Gly Pro Pro Leu
    180                 185                 190

GGA GAA AGT GTG GCC TAT CAT TCT GAG AGT CCC AAC TCA CAG ACA GAT      860
Gly Glu Ser Val Ala Tyr His Ser Glu Ser Pro Asn Ser Gln Thr Asp
195                 200                 205                 210

GTA GGA AGA CCT TTG TCT GGA TCT GGT ATA TCA GCA TTT GTT CAA GCT      908
Val Gly Arg Pro Leu Ser Gly Ser Gly Ile Ser Ala Phe Val Gln Ala
                215                 220                 225

CAC CCT AGC AAC GGA CAG AGA GTG AAC CCC CCA CCA CCA CCT CAA GTA      956
His Pro Ser Asn Gly Gln Arg Val Asn Pro Pro Pro Pro Pro Gln Val
            230                 235                 240

AGG AGT GTT ACT CCT CCA CCA CCT CCA AGA GGC CAG ACT CCC CCT CCA     1004
Arg Ser Val Thr Pro Pro Pro Pro Pro Arg Gly Gln Thr Pro Pro Pro
        245                 250                 255

AGA GGT ACA ACT CCA CCT CCC CCT TCA TGG GAA CCA AAC TCT CAA ACA     1052
Arg Gly Thr Thr Pro Pro Pro Pro Ser Trp Glu Pro Asn Ser Gln Thr
    260                 265                 270

AAG CGC TAT TCT GGA AAC ATG GAA TAC GTA ATC TCC CGA ATC TCT CCT     1100
Lys Arg Tyr S r Gly Asn Met Glu Tyr Val Ile Ser Arg Ile Ser Pro
275                 280                 285                 290
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GTC CCA CCT GGG GCA TGG CAA GAG GGC TAT CCT CCA CCA CCT CTC AAC 1148
Val Pro Pro Gly Ala Trp Gln Glu Gly Tyr Pr Pro Pro Pro Leu Asn
295 300 305

ACT TCC CCC ATG AAT CCT CCT AAT CAA GGA CAG AGA GGC ATT AGT TCT 1196
Thr Ser Pro M t Asn Pro Pro Asn Gln Gly Gln Arg Gly Ile Ser Ser
310 315 320

GTT CCT GTT GGC AGA CAA CCA ATC ATC ATG CAG AGT TCT AGC AAA TTT 1244
Val Pro Val Gly Arg Gln Pro Ile Ile Met Gln Ser Ser Ser Lys Phe
325 330 335

AAC TTT CCA TCA GGG AGA CCT GGA ATG CAG AAT GGT ACT GGA CAA ACT 1292
Asn Phe Pro Ser Gly Arg Pro Gly Met Gln Asn Gly Thr Gly Gln Thr
340 345 350

GAT TTC ATG ATA CAC CAA AAT GTT GTC CCT GCT GGC ACT GTG AAT CGG 1340
Asp Phe Met Ile His Gln Asn Val Val Pro Ala Gly Thr Val Asn Arg
355 360 365 370

CAG CCA CCA CCT CCA TAT CCT CTG ACA GCA GCT AAT GGA CAA AGC CCT 1388
Gln Pro Pro Pro Pro Tyr Pro Leu Thr Ala Ala Asn Gly Gln Ser Pro
375 380 385

TCT GCT TTA CAA ACA GGG GGA TCT GCT GCT CCT TCG TCA TAT ACA AAT 1436
Ser Ala Leu Gln Thr Gly Gly Ser Ala Ala Pro Ser Ser Tyr Thr Asn
390 395 400

GGA AGT ATT CCT CAG TCT ATG ATG GTG CCA AAC AGA AAT AGT CAT AAC 1484
Gly Ser Ile Pro Gln Ser Met Met Val Pro Asn Arg Asn Ser His Asn
405 410 415

ATG GAA CTA TAT AAC ATT AGT GTA CCT GGA CTG CAA ACA AAT TGG CCT 1532
Met Glu Leu Tyr Asn Ile Ser Val Pro Gly Leu Gln Thr Asn Trp Pro
420 425 430

CAG TCA TCT TCT GCT CCA GCC CAG TCA TCC CCG AGC AGT GGG CAT GAA 1580
Gln Ser Ser Ser Ala Pro Ala Gln Ser Ser Pro Ser Ser Gly His Glu
435 440 445 450

ATC CCT ACA TGG CAA CCT AAC ATA CCA GTG AGG TCA AAT TCT TTT AAT 1628
Ile Pro Thr Trp Gln Pro Asn Ile Pro Val Arg Ser Asn Ser Phe Asn
455 460 465

AAC CCA TTA GGA AAT AGA GCA AGT CAC TCT GCT AAT TCT CAG CCT TCT 1676
Asn Pro Leu Gly Asn Arg Ala Ser His Ser Ala Asn Ser Gln Pro Ser
470 475 480

GCT ACA ACA GTC ACT GCA ATT ACA CCA GCT CCT ATT CAA CAG CCT GTG 1724
Ala Thr Thr Val Thr Ala Ile Thr Pro Ala Pro Ile Gln Gln Pro Val
485 490 495

AAA AGT ATG CGT GTA TTA AAA CCA GAG CTA CAG ACT GCT TTA GCA CCT 1772
Lys Ser Met Arg Val Leu Lys Pro Glu Leu Gln Thr Ala Leu Ala Pro
500 505 510

ACA CAC CCT TCT TGG ATA CCA CAG CCA ATT CAA ACT GTT CAA CCC AGT 1820
Thr His Pro Ser Trp Ile Pro Gln Pro Ile Gln Thr Val Gln Pro Ser
515 520 525 530

CCT TTT CCT GAG GGA ACC GCT TCA AAT GTG ACT GTG ATG CCA CCT GTT 1868
Pro Phe Pro Glu Gly Thr Ala Ser Asn Val Thr Val Met Pro Pro Val
535 540 545

GCT GAA GCT CCA AAC TAT CAA GGA CCA CCA CCA CCC TAC CCA AAA CAT 1916
Ala Glu Ala Pro Asn Tyr Gln Gly Pro Pro Pro Pro Tyr Pro Lys His
550 555 560

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CTG CTG CAC CAA AAC CCA TCT GTT CCT CCA TAC  AG TCA ATC AGT AAG     1964
Leu Leu His  ln Asn Pro Ser Val Pro Pro Tyr Glu Ser Ile Ser Lys
        565                 570                 575

CCT AGC AAA GAG GAT CAG CCA AGC TTG CCC AAG GAA GAT GAG AGT GAA     2012
Pro Ser Lys Glu Asp Gln Pro Ser Leu Pro Lys  lu Asp Glu Ser Glu
    580                 585                 590

AAG AGT TAT GAA AAT GTT GAT AGT GGG GAT AAA GAA AAG AAA CAG ATT     2060
Lys Ser Tyr Glu Asn Val Asp Ser Gly Asp Lys Glu Lys Lys Gln Ile
595                 600                 605                 610

ACA ACT TCA CCT ATT ACT GTT AGG AAA AAC AAG AAA GAT GAA GAG CGA     2108
Thr Thr Ser Pro Ile Thr Val Arg Lys Asn Lys Lys Asp Glu Glu Arg
                615                 620                 625

AGG GAA TCT CGT ATT CAA AGT TAT TCT CCT CAA GCA TTT AAA TTC TTT     2156
Arg Glu Ser Arg Ile Gln Ser Tyr Ser Pro Gln Ala Phe Lys Phe Phe
            630                 635                 640

ATG GAG CAA CAT GTA GAA AAT GTA CTC AAA TCT CAT CAG CAG CGT CTA     2204
Met Glu Gln His Val Glu Asn Val Leu Lys Ser His Gln Gln Arg Leu
        645                 650                 655

CAT CGT AAA AAA CAA TTA GAG AAT GAA ATG ATG CGG GTT GGA TTA TCT     2252
His Arg Lys Lys Gln Leu Glu Asn Glu Met Met Arg Val Gly Leu Ser
    660                 665                 670

CAA GAT GCC CAG GAT CAA ATG AGA AAG ATG CTT TGC CAA AAA GAA TCT     2300
Gln Asp Ala Gln Asp Gln Met Arg Lys Met Leu Cys Gln Lys Glu Ser
675                 680                 685                 690

AAT TAC ATC CGT CTT AAA AGG GCT AAA ATG GAC AAG TCT ATG TTT GTG     2348
Asn Tyr Ile Arg Leu Lys Arg Ala Lys Met Asp Lys Ser Met Phe Val
                695                 700                 705

AAG ATA AAG ACA CTA GGA ATA GGA GCA TTT GGT GAA GTC TGT CTA GCA     2396
Lys Ile Lys Thr Leu Gly Ile Gly Ala Phe Gly Glu Val Cys Leu Ala
            710                 715                 720

AGA AAA GTA GAT ACT AAG GCT TTG TAT GCA ACA AAA ACT CTT CGA AAG     2444
Arg Lys Val Asp Thr Lys Ala Leu Tyr Ala Thr Lys Thr Leu Arg Lys
        725                 730                 735

AAA GAT GTT CTT CTT CGA AAT CAA GTC GCT CAT GTT AAG GCT GAG AGA     2492
Lys Asp Val Leu Leu Arg Asn Gln Val Ala His Val Lys Ala Glu Arg
    740                 745                 750

GAT ATC CTG GCT GAA GCT GAC AAT GAA TGG GTA GTT CGT CTA TAT TAT     2540
Asp Ile Leu Ala Glu Ala Asp Asn Glu Trp Val Val Arg Leu Tyr Tyr
755                 760                 765                 770

TCA TTC CAA GAT AAG GAC AAT TTA TAC TTT GTA ATG GAC TAC ATT CCT     2588
Ser Phe Gln Asp Lys Asp Asn Leu Tyr Phe Val Met Asp Tyr Ile Pro
                775                 780                 785

GGG GGT GAT ATG ATG AGC CTA TTA ATT AGA ATG GGC ATC TTT CCA GAA     2636
Gly Gly Asp Met Met Ser Leu Leu Ile Arg Met Gly Ile Phe Pro Glu
            790                 795                 800

AGT CTG GCA CGA TTC TAC ATA GCA GAA CTT ACC TGT GCA GTT GAA AGT     2684
Ser Leu Ala Arg Phe Tyr Ile Ala Glu Leu Thr Cys Ala Val Glu Ser
        805                 810                 815

GTT CAT AAA ATG GGT TTT ATT CAT AGA GAT ATT AAA CCT GAT AAT ATT     2732
Val His Lys Met Gly Ph  Il  His Arg Asp Ile Lys Pro Asp Asn Ile
    820                 825                 830
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TTG ATT GAT CGT GAT GGT CAT ATT AAA TTG ACT GAC TTT GGC CTC TGC 2780
Leu Ile Asp Arg Asp Gly His Ile Lys Leu Thr Asp Phe Gly Leu Cys
835 840 845 850

ACT GGC TTC AGA TGG ACA CAC GAT TCT AAG TAC TAT CAG AGT GGT GAC 2828
Thr Gly Phe Arg Trp Thr His Asp Ser Lys Tyr Tyr Gln Ser Gly Asp
855 860 865

CAT CCA CGG CAA GAT AGC ATG GAT TTC AGT AAT GAA TGG GGG GAT CCC 2876
His Pro Arg Gln Asp Ser Met Asp Phe Ser Asn Glu Trp Gly Asp Pro
870 875 880

TCA AGC TGT CGA TGT GGA GAC AGA CTG AAG CCA TTA GAG CGG AGA GCT 2924
Ser Ser Cys Arg Cys Gly Asp Arg Leu Lys Pro Leu Glu Arg Arg Ala
885 890 895

GCA CGC CAG CAC CAG CGA TGT CTA GCA CAT TCT TTG GTT GGG ACT CCC 2972
Ala Arg Gln His Gln Arg Cys Leu Ala His Ser Leu Val Gly Thr Pro
900 905 910

AAT TAT ATT GCA CCT GAA GTG TTG CTA CGA ACA GGA TAC ACA CAG TTG 3020
Asn Tyr Ile Ala Pro Glu Val Leu Leu Arg Thr Gly Tyr Thr Gln Leu
915 920 925 930

TGT GAT TGG TGG AGT GTT GGT GTT ATT CTT TTT GAA ATG TTG GTG GGA 3068
Cys Asp Trp Trp Ser Val Gly Val Ile Leu Phe Glu Met Leu Val Gly
935 940 945

CAA CCT CCT TTC TTG GCA CAA ACA CCA TTA GAA ACA CAA ATG AAG GTT 3116
Gln Pro Pro Phe Leu Ala Gln Thr Pro Leu Glu Thr Gln Met Lys Val
950 955 960

ATC AAC TGG CAA ACA TCT CTT CAC ATT CCA CCA CAA GCT AAA CTC AGT 3164
Ile Asn Trp Gln Thr Ser Leu His Ile Pro Pro Gln Ala Lys Leu Ser
965 970 975

CCT GAA GCT TCT GAT CTT ATT ATT AAA CTT TGC CGA GGA CCC GAA GAT 3212
Pro Glu Ala Ser Asp Leu Ile Ile Lys Leu Cys Arg Gly Pro Glu Asp
980 985 990

CGC TTA GGC AAG AAT GGT GCT GAT GAA ATA AAA GCT CAT CCA TTT TTT 3260
Arg Leu Gly Lys Asn Gly Ala Asp Glu Ile Lys Ala His Pro Phe Phe
995 1000 1005 1010

AAA ACA ATT GAC TTC TCC AGT GAC CTG AGA CAG CAG TCT GCT TCA TAC 3308
Lys Thr Ile Asp Phe Ser Ser Asp Leu Arg Gln Gln Ser Ala Ser Tyr
1015 1020 1025

ATT CCT AAA ATC ACA CAC CCA ACA GAT ACA TCA AAT TTT GAT CCT GTT 3356
Ile Pro Lys Ile Thr His Pro Thr Asp Thr Ser Asn Phe Asp Pro Val
1030 1035 1040

GAT CCT GAT AAA TTA TGG AGT GAT GAT AAC GAG GAA GAA AAT GTA AAT 3404
Asp Pro Asp Lys Leu Trp Ser Asp Asp Asn Glu Glu Glu Asn Val Asn
1045 1050 1055

GAC ACT CTC AAT GGA TGG TAT AAA AAT GGA AAG CAT CCT GAA CAT GCA 3452
Asp Thr Leu Asn Gly Trp Tyr Lys Asn Gly Lys His Pro Glu His Ala
1060 1065 1070

TTC TAT GAA TTT ACC TTC CGA AGG TTT TTT GAT GAC AAT GGC TAC CCA 3500
Phe Tyr Glu Phe Thr Phe Arg Arg Phe Phe Asp Asp Asn Gly Tyr Pro
1075 1080 1085 1090

TAT AAT TAT CCG AAG CCT ATT GAA TAT GAA TAC ATT AAT TCA CAA GGC 3548
Tyr Asn Tyr Pro Lys Pro Ile Glu Tyr Glu Tyr Ile Asn Ser Gln Gly
1095 1100 1105

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TCA GAG CAG CAG TCG GAT GAA GAT GAT CAA AAC ACA GGC TCA GAG ATT      3596
Ser Glu Gln  ln Ser Asp Glu Asp Asp Gln Asn Thr Gly S r Glu Il
            1110                1115                1120

AAA AAT CGC GAT CTA GTA TAT GTT TAA CACACTAGTA AATAAATGTA            3643
Lys Asn Arg Asp Leu Val Tyr Val  *
        1125                1130

ATGAGGATTT GTAAAAGGGC CTGAAATGCG AGGTGTTTTG AGGTTCTGAG AGTAAAATTA    3703

TGCAAATATG ACAGAGCTAT ATATGTGTGC TCTGTGTACA ATATTTTATT TTCCTAAATT    3763

ATGGGAAATC CTTTTAAAAT GTTAATTTAT TCCAGCCGTT TAAATCAGTA TTTAGAAAAA    3823

AATTGTTATA AGGAAAGTAA ATTATGAACT GAATATTATA GTCAGTTCTT GGTACTTAAA    3883

GTACTTAAAA TAAGTAGTGC TTTGTTTAAA AGGAGAAACC TGGTATCTAT TTGTATATAT    3943

GCTAAATAAT TTTAAAATAC AAGAGTTTTT GAAATTTTTT T                        3984
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1131 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Met Lys Arg Ser Glu Lys Pro Glu Gly Tyr Arg Gln Met Arg Pro Lys
 1               5                  10                  15

Thr Phe Pro Ala Ser Asn Tyr Thr Val Ser Ser Arg Gln Met Leu Gln
            20                  25                  30

Glu Ile Arg Glu Ser Leu Arg Asn Leu Ser Lys Pro Ser Asp Ala Ala
        35                  40                  45

Lys Ala Glu His Asn Met Ser Lys Met Ser Thr Glu Asp Pro Arg Gln
    50                  55                  60

Val Arg Asn Pro Pro Lys Phe Gly Thr His His Lys Ala Leu Gln Glu
65                  70                  75                  80

Ile Arg Asn Ser Leu Leu Pro Phe Ala Asn Glu Thr Asn Ser Ser Arg
                85                  90                  95

Ser Thr Ser Glu Val Asn Pro Gln Met Leu Gln Asp Leu Gln Ala Ala
            100                 105                 110

Gly Phe Asp Glu Asp Met Val Ile Gln Ala Leu Gln Lys Thr Asn Asn
        115                 120                 125

Arg Ser Ile Glu Ala Ala Ile Glu Phe Ile Ser Lys Met Ser Tyr Gln
    130                 135                 140

Asp Pro Arg Arg Glu Gln Met Ala Ala Ala Ala Ala Arg Pro Ile Asn
145                 150                 155                 160

Ala Ser Met Lys Pro Gly Asn Val Gln Gln Ser Val Asn Arg Lys Gln
                165                 170                 175

Ser Trp Lys Gly Ser Lys Glu Ser Leu Val Pro Gln Arg His Gly Pro
            180                 185                 190
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Pro Leu Gly Glu Ser Val Ala Tyr His S r Glu Ser Pro Asn Ser Gln
195 200 205

Thr Asp Val Gly Arg Pro Leu Ser Gly Ser Gly Ile Ser Ala Phe Val
210 215 220

Gln Ala His Pro Ser Asn Gly Gln Arg Val Asn Pro Pro Pro Pro Pro
225 230 235 240

Gln Val Arg Ser Val Thr Pro Pro Pro Pro Pro Arg Gly Gln Thr Pro
245 250 255

Pro Pro Arg Gly Thr Thr Pro Pro Pro Pro Ser Trp Glu Pro Asn Ser
260 265 270

Gln Thr Lys Arg Tyr Ser Gly Asn Met Glu Tyr Val Ile Ser Arg Ile
275 280 285

Ser Pro Val Pro Pro Gly Ala Trp Gln Glu Gly Tyr Pro Pro Pro Pro
290 295 300

Leu Asn Thr Ser Pro Met Asn Pro Pro Asn Gln Gly Gln Arg Gly Ile
305 310 315 320

Ser Ser Val Pro Val Gly Arg Gln Pro Ile Ile Met Gln Ser Ser Ser
325 330 335

Lys Phe Asn Phe Pro Ser Gly Arg Pro Gly Met Gln Asn Gly Thr Gly
340 345 350

Gln Thr Asp Phe Met Ile His Gln Asn Val Val Pro Ala Gly Thr Val
355 360 365

Asn Arg Gln Pro Pro Pro Pro Tyr Pro Leu Thr Ala Ala Asn Gly Gln
370 375 380

Ser Pro Ser Ala Leu Gln Thr Gly Gly Ser Ala Ala Pro Ser Ser Tyr
385 390 395 400

Thr Asn Gly Ser Ile Pro Gln Ser Met Met Val Pro Asn Arg Asn Ser
405 410 415

His Asn Met Glu Leu Tyr Asn Ile Ser Val Pro Gly Leu Gln Thr Asn
420 425 430

Trp Pro Gln Ser Ser Ser Ala Pro Ala Gln Ser Ser Pro Ser Ser Gly
435 440 445

His Glu Ile Pro Thr Trp Gln Pro Asn Ile Pro Val Arg Ser Asn Ser
450 455 460

Phe Asn Asn Pro Leu Gly Asn Arg Ala Ser His Ser Ala Asn Ser Gln
465 470 475 480

Pro Ser Ala Thr Thr Val Thr Ala Ile Thr Pro Ala Pro Ile Gln Gln
485 490 495

Pro Val Lys Ser Met Arg Val Leu Lys Pro Glu Leu Gln Thr Ala Leu
500 505 510

Ala Pro Thr His Pro Ser Trp Ile Pro Gln Pro Ile Gln Thr Val Gln
515 520 525

Pro Ser Pro Phe Pro Glu Gly Thr Ala Ser Asn Val Thr Val Met Pro
530 535 540

Pro Val Ala Glu Ala Pr Asn Tyr Gln Gly Pro Pro Pro Pro Tyr Pro

545 550 555 560

Lys His Leu Leu His Gln Asn Pro Ser Val Pro Pro Tyr Glu S r Ile
565 570 575

Ser Lys Pro Ser Lys Glu Asp Gln Pro Ser Leu Pro Lys Glu Asp Glu
580 585 590

Ser Glu Lys Ser Tyr Glu Asn Val Asp Ser Gly Asp Lys Glu Lys Lys
595 600 605

Gln Ile Thr Thr Ser Pro Ile Thr Val Arg Lys Asn Lys Lys Asp Glu
610 615 620

Glu Arg Arg Glu Ser Arg Ile Gln Ser Tyr Ser Pro Gln Ala Phe Lys
625 630 635 640

Phe Phe Met Glu Gln His Val Glu Asn Val Leu Lys Ser His Gln Gln
645 650 655

Arg Leu His Arg Lys Lys Gln Leu Glu Asn Glu Met Met Arg Val Gly
660 665 670

Leu Ser Gln Asp Ala Gln Asp Gln Met Arg Lys Met Leu Cys Gln Lys
675 680 685

Glu Ser Asn Tyr Ile Arg Leu Lys Arg Ala Lys Met Asp Lys Ser Met
690 695 700

Phe Val Lys Ile Lys Thr Leu Gly Ile Gly Ala Phe Gly Glu Val Cys
705 710 715 720

Leu Ala Arg Lys Val Asp Thr Lys Ala Leu Tyr Ala Thr Lys Thr Leu
725 730 735

Arg Lys Lys Asp Val Leu Leu Arg Asn Gln Val Ala His Val Lys Ala
740 745 750

Glu Arg Asp Ile Leu Ala Glu Ala Asp Asn Glu Trp Val Val Arg Leu
755 760 765

Tyr Tyr Ser Phe Gln Asp Lys Asp Asn Leu Tyr Phe Val Met Asp Tyr
770 775 780

Ile Pro Gly Gly Asp Met Met Ser Leu Leu Ile Arg Met Gly Ile Phe
785 790 795 800

Pro Glu Ser Leu Ala Arg Phe Tyr Ile Ala Glu Leu Thr Cys Ala Val
805 810 815

Glu Ser Val His Lys Met Gly Phe Ile His Arg Asp Ile Lys Pro Asp
820 825 830

Asn Ile Leu Ile Asp Arg Asp Gly His Ile Lys Leu Thr Asp Phe Gly
835 840 845

Leu Cys Thr Gly Phe Arg Trp Thr His Asp Ser Lys Tyr Tyr Gln Ser
850 855 860

Gly Asp His Pro Arg Gln Asp Ser Met Asp Phe Ser Asn Glu Trp Gly
865 870 875 880

Asp Pro Ser Ser Cys Arg Cys Gly Asp Arg Leu Lys Pro Leu Glu Arg
885 890 895

Arg Ala Ala Arg Gln His Gln Arg Cys Leu Ala His Ser Leu Val Gly
900 905 910

Thr Pro Asn Tyr Il Ala Pro Glu Val Leu Leu Arg Thr ly Tyr Thr
        915   920   925

Gln Leu Cys Asp Trp Trp Ser Val Gly Val Ile Leu Phe Glu Met Leu
    930   935   940

Val Gly Gln Pro Pro Phe Leu Ala Gln Thr Pro L u Glu Thr Gln Met
945   950   955   960

Lys Val Ile Asn Trp Gln Thr Ser Leu His Ile Pro Pro Gln Ala Lys
                965   970   975

Leu Ser Pro Glu Ala Ser Asp Leu Ile Ile Lys Leu Cys Arg Gly Pro
            980   985   990

Glu Asp Arg Leu Gly Lys Asn Gly Ala Asp Glu Ile Lys Ala His Pro
        995   1000   1005

Phe Phe Lys Thr Ile Asp Phe Ser Ser Asp Leu Arg Gln Gln Ser Ala
    1010   1015   1020

Ser Tyr Ile Pro Lys Ile Thr His Pro Thr Asp Thr Ser Asn Phe Asp
1025   1030   1035   1040

Pro Val Asp Pro Asp Lys Leu Trp Ser Asp Asp Asn Glu Glu Glu Asn
                1045   1050   1055

Val Asn Asp Thr Leu Asn Gly Trp Tyr Lys Asn Gly Lys His Pro Glu
            1060   1065   1070

His Ala Phe Tyr Glu Phe Thr Phe Arg Arg Phe Phe Asp Asp Asn Gly
        1075   1080   1085

Tyr Pro Tyr Asn Tyr Pro Lys Pro Ile Glu Tyr Glu Tyr Ile Asn Ser
    1090   1095   1100

Gln Gly Ser Glu Gln Gln Ser Asp Glu Asp Asp Gln Asn Thr Gly Ser
1105   1110   1115   1120

Glu Ile Lys Asn Arg Asp Leu Val Tyr Val *
                1125   1130

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3213 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..2889

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTG CAA CAT TCA ATT AAC CGA AAA CAA AGC TGG AAA GGT TCT AAA GAG   48
Val Gln His Ser Ile Asn Arg Lys Gln Ser Trp Lys Gly Ser Lys Glu
  1   5   10   15

TCT CTA GTT CCT CAG AGA CAC GGC CCA TCT CTA GGA GAA AAT GTG GTT   96
Ser Leu Val Pro Gln Arg His Gly Pro Ser Leu Gly Glu Asn Val Val
            20   25   30

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TAT CGT TCT GAA AGC CCC AAC TCA CAG GCG GAT GTA GGA AGA CCT CTG      144
Tyr Arg Ser Glu Ser Pro Asn Ser Gln Ala Asp Val Gly Arg Pro Leu
         35                  40                  45

TCT GGA TCC GGC ATT GCA GCA TTT GCT CAA GCT CAC CCA AGC AAT GGA      192
Ser Gly Ser Gly Ile Ala Ala Ph  Ala Gln Ala His Pr  Ser Asn Gly
     50                  55                  60

CAG AGA GTG AAC CCC CCA CCA CCA CCT CAA GTT AGG AGT GTT ACT CCT      240
Gln Arg Val Asn Pro Pro Pro Pro Pro Gln Val Arg Ser Val Thr Pro
 65                  70                  75                  80

CCA CCA CCT CCG AGA GGC CAG ACC CCA CCT CCC CGA GGC ACC ACT CCC      288
Pro Pro Pro Pro Arg Gly Gln Thr Pro Pro Pro Arg Gly Thr Thr Pro
                 85                  90                  95

CCT CCC CCC TCA TGG GAA CCA AGC TCT CAG ACA AAG CGC TAC TCT GGG      336
Pro Pro Pro Ser Trp Glu Pro Ser Ser Gln Thr Lys Arg Tyr Ser Gly
            100                 105                 110

AAC ATG GAG TAC GTA ATC TCC CGA ATC TCC CCT GTT CCA CCT GGG GCG      384
Asn Met Glu Tyr Val Ile Ser Arg Ile Ser Pro Val Pro Pro Gly Ala
        115                 120                 125

TGG CAG GAG GGG TAC CCT CCA CCA CCT CTT ACC ACT TCT CCC ATG AAT      432
Trp Gln Glu Gly Tyr Pro Pro Pro Pro Leu Thr Thr Ser Pro Met Asn
    130                 135                 140

CCC CCT AGC CAG GCT CAG AGG GCC ATT AGT TCT GTT CCA GTT GGT AGA      480
Pro Pro Ser Gln Ala Gln Arg Ala Ile Ser Ser Val Pro Val Gly Arg
145                 150                 155                 160

CAA CCC ATC ATC ATG CAG AGT ACT AGC AAA TTT AAC TTT ACA CCA GGG      528
Gln Pro Ile Ile Met Gln Ser Thr Ser Lys Phe Asn Phe Thr Pro Gly
                165                 170                 175

CGA CCT GGA GTT CAG AAT GGT GGT GGT CAG TCT GAT TTT ATC GTG CAC      576
Arg Pro Gly Val Gln Asn Gly Gly Gly Gln Ser Asp Phe Ile Val His
            180                 185                 190

CAA AAT GTC CCC ACT GGT TCT GTG ACT CGG CAG CCA CCA CCT CCA TAT      624
Gln Asn Val Pro Thr Gly Ser Val Thr Arg Gln Pro Pro Pro Pro Tyr
        195                 200                 205

CCT CTG ACC CCA GCT AAT GGA CAA AGC CCC TCT GCT TTA CAA ACA GGG      672
Pro Leu Thr Pro Ala Asn Gly Gln Ser Pro Ser Ala Leu Gln Thr Gly
    210                 215                 220

GCT TCT GCT GCT CCA CCA TCA TTC GCC AAT GGA AAC GTT CCT CAG TCG      720
Ala Ser Ala Ala Pro Pro Ser Phe Ala Asn Gly Asn Val Pro Gln Ser
225                 230                 235                 240

ATG ATG GTG CCC AAC AGG AAC AGT CAT AAC ATG GAG CTT TAT AAT ATT      768
Met Met Val Pro Asn Arg Asn Ser His Asn Met Glu Leu Tyr Asn Ile
                245                 250                 255

AAT GTC CCT GGA CTG CAA ACA GCC TGG CCC CAG TCG TCT TCT GCT CCT      816
Asn Val Pro Gly Leu Gln Thr Ala Trp Pro Gln Ser Ser Ser Ala Pro
            260                 265                 270

GCG CAG TCA TCC CCA AGC GGT GGG CAT GAA ATT CCT ACA TGG CAA CCT      864
Ala Gln Ser Ser Pro Ser Gly Gly His Glu Ile Pro Thr Trp Gln Pro
        275                 280                 285

AAC ATA CCA GTG AGG TCA AAT TCT TTT AAT AAC CCA TTA GGA AGT AGA      912
Asn Ile Pro Val Arg Ser Asn Ser Phe Asn Asn Pro Leu Gly Ser Arg
    290                 295                 300
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GCA AGT CAC TCT GCT AAT TCT CAG CCT TCT GCC ACT ACA GTC ACT GCC 960
Ala Ser His Ser Ala Asn Ser Gln Pro S r Ala Thr Thr Val Thr Ala
305 310 315 320

ATC ACA CCC GCT CCT ATT CAA CAG CCC GTG AAA AGC ATG CGC GTC CTG 1008
Ile Thr Pro Ala Pro Ile Gln Gln Pro Val Lys Ser Met Arg Val Leu
325 330 335

AAA CCA GAG CTG CAG ACT GCT TTA GCC CCA ACC CAT CCT TCT TGG ATG 1056
Lys Pro Glu Leu Gln Thr Ala Leu Ala Pro Thr His Pro Ser Trp Met
340 345 350

CCA CAG CCA GTT CAG ACT GTT CAG CCT ACC CCT TTT TCT GAG GGT ACA 1104
Pro Gln Pro Val Gln Thr Val Gln Pro Thr Pro Phe Ser Glu Gly Thr
355 360 365

GCT TCA AGT GTG CCT GTC ATC CCA CCT GTT GCT GAA GCT CCA AGC TAT 1152
Ala Ser Ser Val Pro Val Ile Pro Pro Val Ala Glu Ala Pro Ser Tyr
370 375 380

CAA GGT CCA CCA CCG CCT TAT CCA AAA CAT CTG CTA CAC CAA AAC CCA 1200
Gln Gly Pro Pro Pro Pro Tyr Pro Lys His Leu Leu His Gln Asn Pro
385 390 395 400

TCT GTC CCT CCA TAT GAG TCA GTA AGT AAG CCC TGC AAA GAT GAA CAG 1248
Ser Val Pro Pro Tyr Glu Ser Val Ser Lys Pro Cys Lys Asp Glu Gln
405 410 415

CCT AGC TTA CCC AAG GAA GAT GAT AGT GAG AAG AGT GCG GAC AGT GGT 1296
Pro Ser Leu Pro Lys Glu Asp Asp Ser Glu Lys Ser Ala Asp Ser Gly
420 425 430

GAC TCT GGG GAT AAA GAA AAG AAA CAG ATT ACA ACT TCA CCT ATC ACT 1344
Asp Ser Gly Asp Lys Glu Lys Lys Gln Ile Thr Thr Ser Pro Ile Thr
435 440 445

GTT CGG AAA AAC AAG AAA GAT GAA GAA CGA AGA GAG TCT CGG ATT CAG 1392
Val Arg Lys Asn Lys Lys Asp Glu Glu Arg Arg Glu Ser Arg Ile Gln
450 455 460

AGT TAC TCC CCA CAG GCC TTT AAG TTC TTC ATG GAG CAG CAC GTA GAG 1440
Ser Tyr Ser Pro Gln Ala Phe Lys Phe Phe Met Glu Gln His Val Glu
465 470 475 480

AAC GTC CTG AAG TCT CAT CAG CAG CGT CTG CAT CGG AAG AAG CAG CTA 1488
Asn Val Leu Lys Ser His Gln Gln Arg Leu His Arg Lys Lys Gln Leu
485 490 495

GAA AAT GAA ATG ATG CGG GTT GGA TTA TCT CAA GAT GCC CAG GAT CAA 1536
Glu Asn Glu Met Met Arg Val Gly Leu Ser Gln Asp Ala Gln Asp Gln
500 505 510

ATG AGA AAG ATG CTT TGC CAG AAA GAG TCT AAC TAT ATT CGT CTT AAA 1584
Met Arg Lys Met Leu Cys Gln Lys Glu Ser Asn Tyr Ile Arg Leu Lys
515 520 525

AGG GCT AAA ATG GAC AAG TCT ATG TTT GTA AAG ATA AAG ACA TTA GGA 1632
Arg Ala Lys Met Asp Lys Ser Met Phe Val Lys Ile Lys Thr Leu Gly
530 535 540

ATA GGA GCG TTT GGT GAA GTC TGT CTA GCA AGA AAA GTC GAT ACT AAA 1680
Ile Gly Ala Phe Gly Glu Val Cys Leu Ala Arg Lys Val Asp Thr Lys
545 550 555 560

GCT TTG TAT GCA ACA AAG ACT CTT CGA AAG AAA GAC GTT CTG CTC CGA 1728
Ala L u Tyr Ala Thr Lys Thr Leu Arg Lys Lys Asp Val L u Leu Arg
565 570 575

AAT CAG GTG GCT CAT GTG AAA GCG GAG AGG GAT ATC CTA GCA GAA GCC 1776
Asn Gln Val Ala His Val Lys Ala Glu Arg Asp Ile Leu Ala Glu Ala
580 585 590

GAC AAT GA TGG TG GTC CGC CTG TAC TAC TCT TTC CAG GAC AAG GAC 1824
Asp Asn lu Trp Val Val Arg Leu Tyr Tyr Ser Ph Gln Asp Lys Asp
595 600 605

AAC TTG TAC TTT GTG ATG GAC TAC ATT CCT GGG GGG GAT ATG ATG AGC 1872
Asn Leu Tyr Phe Val Met Asp Tyr Ile Pro Gly Gly Asp Met Met Ser
610 615 620

CTA TTA ATT AGA ATG GGC ATC TTT CCT GAA AAT CTG GCA CGA TTC TAC 1920
Leu Leu Ile Arg Met Gly Ile Phe Pro Glu Asn Leu Ala Arg Phe Tyr
625 630 635 640

ATA GCA GAA CTT ACC TGT GCA GTT GAA AGT GTT CAT AAA ATG GGT TTT 1968
Ile Ala Glu Leu Thr Cys Ala Val Glu Ser Val His Lys Met Gly Phe
645 650 655

ATT CAT AGA GAT ATT AAA CCT GAT AAC ATT TTG ATT GAC CGT GAT GGC 2016
Ile His Arg Asp Ile Lys Pro Asp Asn Ile Leu Ile Asp Arg Asp Gly
660 665 670

CAT ATT AAA TTG ACT GAC TTT GGC TTG TGC ACT GGC TTC AGA TGG ACA 2064
His Ile Lys Leu Thr Asp Phe Gly Leu Cys Thr Gly Phe Arg Trp Thr
675 680 685

CAT GAC TCC AAG TAC TAC CAG AGT GGG GAT CAC CCA CGG CAA GAT AGC 2112
His Asp Ser Lys Tyr Tyr Gln Ser Gly Asp His Pro Arg Gln Asp Ser
690 695 700

ATG GAT TTC AGT AAC GAA TGG GGA GAT CCT TCC AAT TGT CGG TGT GGG 2160
Met Asp Phe Ser Asn Glu Trp Gly Asp Pro Ser Asn Cys Arg Cys Gly
705 710 715 720

GAC AGA CTG AAG CCA CTG GAG CGG AGA GCT GCT CGC CAG CAC CAG CGA 2208
Asp Arg Leu Lys Pro Leu Glu Arg Arg Ala Ala Arg Gln His Gln Arg
725 730 735

TGT CTA GCC CAT TCT CTG GTT GGG ACT CCC AAT TAT ATT GCA CCT GAA 2256
Cys Leu Ala His Ser Leu Val Gly Thr Pro Asn Tyr Ile Ala Pro Glu
740 745 750

GTG CTA CTG CGA ACA GGA TAT ACA CAG CTG TGT GAC TGG TGG AGT GTT 2304
Val Leu Leu Arg Thr Gly Tyr Thr Gln Leu Cys Asp Trp Trp Ser Val
755 760 765

GGT GTT ATT CTT TGT GAA ATG TTG GTG GGA CAA CCT CCT TTC TTG GCA 2352
Gly Val Ile Leu Cys Glu Met Leu Val Gly Gln Pro Pro Phe Leu Ala
770 775 780

CAA ACC CCA TTA GAA ACA CAA ATG AAG GTT ATC ATC TGG CAA ACT TCT 2400
Gln Thr Pro Leu Glu Thr Gln Met Lys Val Ile Ile Trp Gln Thr Ser
785 790 795 800

CTA CAC ATC CCT CCT CAA GCT AAG CTG AGT CCT GAA GCC TCT GAC CTC 2448
Leu His Ile Pro Pro Gln Ala Lys Leu Ser Pro Glu Ala Ser Asp Leu
805 810 815

ATT ATC AAA CTG TGT CGA GGA CCA GAA GAC CGC CTC GGC AAG AAC GGT 2496
Ile Ile Lys Leu Cys Arg Gly Pro Glu Asp Arg Leu Gly Lys Asn Gly
820 825 830

GCT GAT GAG ATA AAG GCT CAT CCA TTT TTT AAG ACC ATC GAT TTC TCT 2544
Ala Asp Glu Ile Lys Ala His Pro Phe Phe Lys Thr Ile Asp Phe Ser
835 840 845

AGT GAT CTG AGA CAG CAG TCT GCT TCA TAC ATC CCT AAA ATC ACG CAT 2592
S r Asp Leu Arg Gln Gln Ser Ala Ser Tyr Ile Pro Lys Ile Thr His
850 855 860

CCA ACA GAT ACA TCC AAT TTC GAC CCT GTT GAT CCT GAT AAA TTG TGG 2640
Pro Thr Asp Thr Ser Asn Phe Asp Pro Val Asp Pro Asp Lys Leu Trp
865 870 875 880

AGC GAT GGC AGC GAG GAG GAA AAT ATC AGT GAC ACT CTG AGC GGA TGG 2688
Ser Asp Gly Ser Glu Glu Glu Asn Ile Ser Asp Thr Leu Ser Gly Trp
885 890 895

TAT AAA [redacted] 2736
Tyr Lys Asn Gly Lys His Pro Glu His Ala Phe Tyr Glu Phe Thr Phe
900 905 910

CGG AGG TTT TTT GAT GAC AAT GGC TAC CCA TAT AAT TAT CCA AAG CCT 2784
Arg Arg Phe Phe Asp Asp Asn Gly Tyr Pro Tyr Asn Tyr Pro Lys Pro
915 920 925

ATT GAG TAT GAA TAC ATT CAT TCA CAG GGC TCA GAA CAA CAG TCT GAT 2832
Ile Glu Tyr Glu Tyr Ile His Ser Gln Gly Ser Glu Gln Gln Ser Asp
930 935 940

GAA GAT GAT CAA CAC ACA AGC TCC GAT GGA AAC AAC CGA GAT CTA GTG 2880
Glu Asp Asp Gln His Thr Ser Ser Asp Gly Asn Asn Arg Asp Leu Val
945 950 955 960

TAT GTT TAA TAAACTAGGA GATCATTGTA AGAATTTGCA AGAGGCCTGA 2929
Tyr Val *

AGTGCAGGGG TTTTTGAAGT TTTGAGAAAA TTATGCAAAT GTGACAGAGT TTGTGTGCTC 2989

TGTGTACAAT ATTTTATTTT CCTAAGTTAT GGGAAATTGT TTTAAAATGT TAATTTATTC 3049

CACCCTTTTA ATTCAGTAAT TTAGAAAAAA TTGTTATAAG GAAAGTAAAT TATGAACTGA 3109

GTATTATAGT CAATTCTTGG TACTTAAAGT ACTTAAAAAG AGAAGCCTGG TATCTTTTGT 3169

ATATATAATA AATAATTTTA AAATCCCAAA AAAAAAAAAA AAAA 3213

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 963 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Val Gln His Ser Ile Asn Arg Lys Gln Ser Trp Lys Gly Ser Lys Glu
1 5 10 15

Ser Leu Val Pro Gln Arg His Gly Pro Ser Leu Gly Glu Asn Val Val
20 25 30

Tyr Arg Ser Glu Ser Pro Asn Ser Gln Ala Asp Val Gly Arg Pro Leu
35 40 45

Ser Gly Ser Gly Ile Ala Ala Phe Ala Gln Ala His Pro Ser Asn Gly
50 55 60

Gln Arg Val Asn Pro Pro Pro Pro Pro Gln Val Arg Ser Val Thr Pro

65 70 75 80

Pro Pro Pro Pro Arg Gly Gln Thr Pro Pro Pro Arg Gly Thr Thr Pro
85 90 95

Pro Pro Pro S r Trp lu Pro Ser S r Gln Thr Lys Arg Tyr Ser ly
100 105 110

Asn Met Glu Tyr Val Ile Ser Arg Ile Ser Pro Val Pro Pro Gly Ala
115 120 125

Trp Gln Glu Gly Tyr Pro Pro Pro Pro Leu Thr Thr Ser Pro Met Asn
130 135 140

Pro Pro Ser Gln Ala Gln Arg Ala Ile Ser Ser Val Pro Val Gly Arg
145 150 155 160

Gln Pro Ile Ile Met Gln Ser Thr Ser Lys Phe Asn Phe Thr Pro Gly
165 170 175

Arg Pro Gly Val Gln Asn Gly Gly Gly Gln Ser Asp Phe Ile Val His
180 185 190

Gln Asn Val Pro Thr Gly Ser Val Thr Arg Gln Pro Pro Pro Pro Tyr
195 200 205

Pro Leu Thr Pro Ala Asn Gly Gln Ser Pro Ser Ala Leu Gln Thr Gly
210 215 220

Ala Ser Ala Ala Pro Pro Ser Phe Ala Asn Gly Asn Val Pro Gln Ser
225 230 235 240

Met Met Val Pro Asn Arg Asn Ser His Asn Met Glu Leu Tyr Asn Ile
245 250 255

Asn Val Pro Gly Leu Gln Thr Ala Trp Pro Gln Ser Ser Ser Ala Pro
260 265 270

Ala Gln Ser Ser Pro Ser Gly Gly His Glu Ile Pro Thr Trp Gln Pro
275 280 285

Asn Ile Pro Val Arg Ser Asn Ser Phe Asn Asn Pro Leu Gly Ser Arg
290 295 300

Ala Ser His Ser Ala Asn Ser Gln Pro Ser Ala Thr Thr Val Thr Ala
305 310 315 320

Ile Thr Pro Ala Pro Ile Gln Gln Pro Val Lys Ser Met Arg Val Leu
325 330 335

Lys Pro Glu Leu Gln Thr Ala Leu Ala Pro Thr His Pro Ser Trp Met
340 345 350

Pro Gln Pro Val Gln Thr Val Gln Pro Thr Pro Phe Ser Glu Gly Thr
355 360 365

Ala Ser Ser Val Pro Val Ile Pro Pro Val Ala Glu Ala Pro Ser Tyr
370 375 380

Gln Gly Pro Pro Pro Pro Tyr Pro Lys His Leu Leu His Gln Asn Pro
385 390 395 400

Ser Val Pro Pro Tyr Glu Ser Val Ser Lys Pro Cys Lys Asp Glu Gln
405 410 415

Pro Ser Leu Pro Lys Glu Asp Asp S r Glu Lys Ser Ala Asp Ser Gly
420 425 430

Asp Ser ly Asp Lys Glu Lys Lys Gln Il Thr Thr Ser Pro Ile Thr
435 440 445

Val Arg Lys Asn Lys Lys Asp Glu Glu Arg Arg Glu Ser Arg Ile Gln
450 455 460

Ser Tyr Ser Pro Gln Ala Phe Lys Phe Phe Met Glu Gln His Val Glu
465 470 475 480

~~Asn Val Leu Lys Ser His Gln Gln Arg Leu His Arg Lys~~ Lys ~~Gln Leu~~
485 490 495

Glu Asn Glu Met Met Arg Val Gly Leu Ser Gln Asp Ala Gln Asp Gln
500 505 510

Met Arg Lys Met Leu Cys Gln Lys Glu Ser Asn Tyr Ile Arg Leu Lys
515 520 525

Arg Ala Lys Met Asp Lys Ser Met Phe Val Lys Ile Lys Thr Leu Gly
530 535 540

Ile Gly Ala Phe Gly Glu Val Cys Leu Ala Arg Lys Val Asp Thr Lys
545 550 555 560

Ala Leu Tyr Ala Thr Lys Thr Leu Arg Lys Lys Asp Val Leu Leu Arg
565 570 575

Asn Gln Val Ala His Val Lys Ala Glu Arg Asp Ile Leu Ala Glu Ala
580 585 590

Asp Asn Glu Trp Val Val Arg Leu Tyr Tyr Ser Phe Gln Asp Lys Asp
595 600 605

Asn Leu Tyr Phe Val Met Asp Tyr Ile Pro Gly Gly Asp Met Met Ser
610 615 620

Leu Leu Ile Arg Met Gly Ile Phe Pro Glu Asn Leu Ala Arg Phe Tyr
625 630 635 640

Ile Ala Glu Leu Thr Cys Ala Val Glu Ser Val His Lys Met Gly Phe
645 650 655

Ile His Arg Asp Ile Lys Pro Asp Asn Ile Leu Ile Asp Arg Asp Gly
660 665 670

His Ile Lys Leu Thr Asp Phe Gly Leu Cys Thr Gly Phe Arg Trp Thr
675 680 685

His Asp Ser Lys Tyr Tyr Gln Ser Gly Asp His Pro Arg Gln Asp Ser
690 695 700

Met Asp Phe Ser Asn Glu Trp Gly Asp Pro Ser Asn Cys Arg Cys Gly
705 710 715 720

Asp Arg Leu Lys Pro Leu Glu Arg Arg Ala Ala Arg Gln His Gln Arg
725 730 735

Cys Leu Ala His Ser Leu Val Gly Thr Pro Asn Tyr Ile Ala Pro Glu
740 745 750

Val Leu Leu Arg Thr Gly Tyr Thr Gln Leu Cys Asp Trp Trp Ser Val
755 760 765

Gly Val Ile Leu Cys Glu Met Leu Val Gly Gln Pro Pro Phe Leu Ala
770 775 780

Gln Thr Pro L u Glu Thr Gln Met Lys Val Ile Ile Trp Gln Thr Ser

```
785                 790                 795                 800

L u His Ile Pro Pro Gln Ala Lys Leu Ser Pro Glu Ala Ser Asp Leu
                805                 810                 815

Il  Ile Lys Leu Cys Arg Gly Pro Glu Asp Arg Leu Gly Lys Asn Gly
            820                 825                 830

Ala Asp Glu Ile Lys Ala His Pro Phe Phe Lys Thr Ile Asp Phe Ser
        835                 840                 845

Ser Asp Leu Arg Gln Gln Ser Ala Ser Tyr Ile Pro Lys Ile Thr His
    850                 855                 860

Pro Thr Asp Thr Ser Asn Phe Asp Pro Val Asp Pro Asp Lys Leu Trp
865                 870                 875                 880

Ser Asp Gly Ser Glu Glu Glu Asn Ile Ser Asp Thr Leu Ser Gly Trp
                885                 890                 895

Tyr Lys Asn Gly Lys His Pro Glu His Ala Phe Tyr Glu Phe Thr Phe
            900                 905                 910

Arg Arg Phe Phe Asp Asp Asn Gly Tyr Pro Tyr Asn Tyr Pro Lys Pro
        915                 920                 925

Ile Glu Tyr Glu Tyr Ile His Ser Gln Gly Ser Glu Gln Gln Ser Asp
    930                 935                 940

Glu Asp Asp Gln His Thr Ser Ser Asp Gly Asn Asn Arg Asp Leu Val
945                 950                 955                 960

Tyr Val  *
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3155 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..2943

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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ATG AGA GCC ACC CCG AAG TTT GGA CCT TAT CAA AAA GCT CTC AGG GAA      48
Met Arg Ala Thr Pro Lys Phe Gly Pro Tyr Gln Lys Ala Leu Arg Glu
  1               5                  10                  15

ATC CGA TAT TCC CTC CTG CCT TTT GCC AAC GAG TCA GGC ACT TCG GCA      96
Ile Arg Tyr Ser Leu Leu Pro Phe Ala Asn Glu Ser Gly Thr Ser Ala
            20                  25                  30

GCT GCA GAG GTG AAC CGG CAG ATG CTT CAG GAG TTG GTG AAT GCG GCA     144
Ala Ala Glu Val Asn Arg Gln Met Leu Gln Glu Leu Val Asn Ala Ala
        35                  40                  45

TGT GAC CAG GAG ATG GCT GGC AGA GCG CTC ACG CAG ACG GGC AGT AGG     192
Cys Asp Gln Glu Met Ala Gly Arg Ala Leu Thr Gln Thr Gly Ser Arg
```

50 55 60

AGT ATC GAA GCT GCC TTG GAG TAC ATC AGT AAG ATG GGC TAC CTG GAC 240
Ser Ile Glu Ala Ala Leu Glu Tyr Ile Ser Lys Met Gly Tyr L u Asp
65 70 75 80

CCC AGG AAT GAG CAG ATT GTG CGA GTC ATC AAG CAG ACC TCC CCA GGA 288
Pro Arg Asn Glu Gln Ile Val Arg Val Ile Lys Gln Thr Ser Pro Gly
85 90 95

AAG GGC CTG GCG TCC ACC CCG GTG ACT CGG CGG CCC AGT TTC GAG GGC 336
Lys Gly Leu Ala Ser Thr Pro Val Thr Arg Arg Pro Ser Phe Glu Gly
100 105 110

ACA GGG GAA GCA CTC CCA TCC TAC CAC CAG CTG GGT GGT GCA AAC TAC 384
Thr Gly Glu Ala Leu Pro Ser Tyr His Gln Leu Gly Gly Ala Asn Tyr
115 120 125

GAG GGC CCC GCC GCA CTG GAG GAG ATG CCG CGG CAA TAT TTA GAC TTT 432
Glu Gly Pro Ala Ala Leu Glu Glu Met Pro Arg Gln Tyr Leu Asp Phe
130 135 140

CTC TTC CCT GGA GCC GGA GCC GGC ACC CAC GGT GCC CAG GCT CAC CAG 480
Leu Phe Pro Gly Ala Gly Ala Gly Thr His Gly Ala Gln Ala His Gln
145 150 155 160

CAT CCT CCC AAA GGG TAC AGC ACA GCA GTA GAG CCA AGT GCG CAC TTT 528
His Pro Pro Lys Gly Tyr Ser Thr Ala Val Glu Pro Ser Ala His Phe
165 170 175

CCG GGC ACA CAC TAT GGT CGT GGT CAT CTA CTA TCG GAG CAG TCT GGG 576
Pro Gly Thr His Tyr Gly Arg Gly His Leu Leu Ser Glu Gln Ser Gly
180 185 190

TAT GGG GTG CAG CGC AGT TCC TCC TTC CAG AAC AAG ACG CCA CCA GAT 624
Tyr Gly Val Gln Arg Ser Ser Ser Phe Gln Asn Lys Thr Pro Pro Asp
195 200 205

GCC TAT TCC AGC ATG GCC AAG GCC CAG GGT GGC CCT CCC GCC AGC CTC 672
Ala Tyr Ser Ser Met Ala Lys Ala Gln Gly Gly Pro Pro Ala Ser Leu
210 215 220

ACC TTT CCT GCC CAT GCT GGG CTG TAC ACT GCC TCG CAC CAC AAG CCG 720
Thr Phe Pro Ala His Ala Gly Leu Tyr Thr Ala Ser His His Lys Pro
225 230 235 240

GCG GCT ACC CCA CCT GGG GCC CAC CCA TTA CAT GTG TTG GGC ACC CGG 768
Ala Ala Thr Pro Pro Gly Ala His Pro Leu His Val Leu Gly Thr Arg
245 250 255

GGT CCC ACG TTT ACT GGC GAA AGC TCT GCA CAG GCT GTG CTG GCA CCG 816
Gly Pro Thr Phe Thr Gly Glu Ser Ser Ala Gln Ala Val Leu Ala Pro
260 265 270

TCC AGG AAC AGC CTC AAT GCT GAC TTG TAC GAG CTG GGC TCC ACG GTG 864
Ser Arg Asn Ser Leu Asn Ala Asp Leu Tyr Glu Leu Gly Ser Thr Val
275 280 285

CCC TGG TCT GCA GCT CCA CTG GCA CGC CGC GAC TCG CTG CAG AAG CAG 912
Pro Trp Ser Ala Ala Pro Leu Ala Arg Arg Asp Ser Leu Gln Lys Gln
290 295 300

GGT CTA GAA GCC TCG CGG CCG CAT GTG GCT TTT CGG GCT GGC CCC AGC 960
Gly Leu Glu Ala Ser Arg Pro His Val Ala Phe Arg Ala Gly Pro Ser
305 310 315 320

AGG ACC AAC TCC TTC AAC AAC CCA CAA CCT GAG CCC TCA CTG CCC GCC 1008

Arg Thr Asn Ser Ph Asn Asn Pro Gln Pr Glu Pro Ser Leu Pro Ala
325 330 335

CCC AAC ACG GTC ACC GCC GTG ACG GCC CA CAC ATC CTT CAC CCT GTG 1056
Pro Asn Thr Val Thr Ala Val Thr Ala Ala His Ile L u His Pro Val
340 345 350

AAG AGC GTG CGT GTG CTG CGG CCC GAG CCC CAG ACA GCC GTG GGG CCC 1104
Lys Ser Val Arg Val Leu Arg Pro Glu Pro Gln Thr Ala Val Gly Pro
355 360 365

TCG CAC CCC GCC TGG GTG GCT GCG CCC ACA GCA CCT GCC ACT GAG AGC 1152
Ser His Pro Ala Trp Val Ala Ala Pro Thr Ala Pro Ala Thr Glu Ser
370 375 380

CTG GAG ACG AAG GAG GGC AGC GCA GGC CCA CAC CCG CTG GAT GTG GAC 1200
Leu Glu Thr Lys Glu Gly Ser Ala Gly Pro His Pro Leu Asp Val Asp
385 390 395 400

TAT GGC GGC TCC GAG CGC AGG TGC CCA CCG CCT CCG TAT CCA AAG CAC 1248
Tyr Gly Gly Ser Glu Arg Arg Cys Pro Pro Pro Pro Tyr Pro Lys His
405 410 415

TTG CTG CTG CCC AGT AAG TCT GAG CAG TAC AGC GTG GAC CTG GAC AGC 1296
Leu Leu Leu Pro Ser Lys Ser Glu Gln Tyr Ser Val Asp Leu Asp Ser
420 425 430

CTG TGC ACC AGT GTG CAG CAG AGT CTG CGA GGG GGC ACT GAT CTA GAC 1344
Leu Cys Thr Ser Val Gln Gln Ser Leu Arg Gly Gly Thr Asp Leu Asp
435 440 445

GGG AGT GAC AAG AGC CAC AAA GGT GCG AAG GGA GAC AAA GCT GGC AGA 1392
Gly Ser Asp Lys Ser His Lys Gly Ala Lys Gly Asp Lys Ala Gly Arg
450 455 460

GAC AAA AAG CAG ATT CAG ACC TCC CCG GTG CCT GTC CGC AAG AAT AGC 1440
Asp Lys Lys Gln Ile Gln Thr Ser Pro Val Pro Val Arg Lys Asn Ser
465 470 475 480

AGA GAT GAA GAG AAG AGA GAG TCT CGC ATC AAG AGT TAC TCC CCT TAT 1488
Arg Asp Glu Glu Lys Arg Glu Ser Arg Ile Lys Ser Tyr Ser Pro Tyr
485 490 495

GCC TTC AAA TTC TTC ATG GAG CAA CAC GTG GAG AAT GTC ATC AAA ACC 1536
Ala Phe Lys Phe Phe Met Glu Gln His Val Glu Asn Val Ile Lys Thr
500 505 510

TAC CAG CAG AAG GTC AGC CGG AGG CTA CAG CTG GAG CAG GAA ATG GCC 1584
Tyr Gln Gln Lys Val Ser Arg Arg Leu Gln Leu Glu Gln Glu Met Ala
515 520 525

AAA GCT GGG CTC TGT GAG GCC GAG CAG GAG CAG ATG AGG AAG ATC CTC 1632
Lys Ala Gly Leu Cys Glu Ala Glu Gln Glu Gln Met Arg Lys Ile Leu
530 535 540

TAC CAG AAG GAG TCT AAC TAC AAC CGG CTG AAG AGG GCC AAG ATG GAC 1680
Tyr Gln Lys Glu Ser Asn Tyr Asn Arg Leu Lys Arg Ala Lys Met Asp
545 550 555 560

AAG TCC ATG TTT GTG AAA ATC AAG ACT CTA GGC ATC GGT GCC TTT GGG 1728
Lys Ser Met Phe Val Lys Ile Lys Thr Leu Gly Ile Gly Ala Phe Gly
565 570 575

GAA GTG TGC CTC GCT TGT AAG CTG GAC ACT CAC GCT CTG TAC GCC ATG 1776
Glu Val Cys Leu Ala Cys Lys Leu Asp Thr His Ala Leu Tyr Ala M t
580 585 590

AAG ACT CTC AGG AAG AAG GAT GTC CTG AAC CGG AAT CAA GTG GCC CAT 1824
Lys Thr Leu Arg Lys Lys Asp Val L u Asn Arg Asn Gln Val Ala His
595 600 605

GTC AAG GCT GAG AGG GAC ATC CTG GCT GAA GCA GAC AAT GAG TGG GTG 1872
Val Lys Ala Glu Arg Asp Ile Leu Ala Glu Ala Asp Asn Glu Trp Val
610 615 620

GTC AAA CTC TAC TAC TCC TTC CAG GAC AAG GAC AGC CTG TAC TTT GTG 1920
Val Lys Leu Tyr Tyr Ser Phe Gln Asp Lys Asp Ser Leu Tyr Phe Val
625 630 635 640

ATG GAC TAC ATA CCA GGC GGG GAT ATG ATG AGC CTG CTG ATC AGG ATG 1968
Met Asp Tyr Ile Pro Gly Gly Asp Met Met Ser Leu Leu Ile Arg Met
645 650 655

GAG GTC TTC CCT GAG CAC CTG GCC CGC TTC TAC ATT GCA GAG TTG ACC 2016
Glu Val Phe Pro Glu His Leu Ala Arg Phe Tyr Ile Ala Glu Leu Thr
660 665 670

CTG GCC ATT GAA AGT GTC CAC AAG ATG GGC TTT ATC CAC CGG GAC ATC 2064
Leu Ala Ile Glu Ser Val His Lys Met Gly Phe Ile His Arg Asp Ile
675 680 685

AAG CCT GAC AAC ATA CTC ATC GAC CTG GAT GGT CAT ATT AAG CTG ACA 2112
Lys Pro Asp Asn Ile Leu Ile Asp Leu Asp Gly His Ile Lys Leu Thr
690 695 700

GAT TTT GGC CTC TGC ACT GGA TTC AGG TGG ACT CAC AAT TCC AAG TAC 2160
Asp Phe Gly Leu Cys Thr Gly Phe Arg Trp Thr His Asn Ser Lys Tyr
705 710 715 720

TAC CAG AAA GGG AAC CAC ATG AGA CAG GAC AGC ATG GAG CCC GGT GAC 2208
Tyr Gln Lys Gly Asn His Met Arg Gln Asp Ser Met Glu Pro Gly Asp
725 730 735

CTC TGG GAC GAT GTT TCC AAC TGT CGC TGT GGA GAC AGG TTA AAG ACC 2256
Leu Trp Asp Asp Val Ser Asn Cys Arg Cys Gly Asp Arg Leu Lys Thr
740 745 750

CTG GAG CAG AGG GCG CAG AAG CAG CAC CAG AGG TGC CTG GCA CAT TCT 2304
Leu Glu Gln Arg Ala Gln Lys Gln His Gln Arg Cys Leu Ala His Ser
755 760 765

CTT GTC GGG ACA CCA AAT TAC ATC GCT CCG GAG GTG CTT CTC CGC AAA 2352
Leu Val Gly Thr Pro Asn Tyr Ile Ala Pro Glu Val Leu Leu Arg Lys
770 775 780

GGG TAC ACG CAG CTC TGT GAC TGG TGG AGC GTC GGT GTG ATT CTC TTT 2400
Gly Tyr Thr Gln Leu Cys Asp Trp Trp Ser Val Gly Val Ile Leu Phe
785 790 795 800

GAG ATG CTG GTT GGG CAG CCG CCT TTC TTG GCC CCC ACC CCC ACA GAG 2448
Glu Met Leu Val Gly Gln Pro Pro Phe Leu Ala Pro Thr Pro Thr Glu
805 810 815

ACG CAG CTG AAG GTG ATC AAC TGG GAG AGC ACG CTG CAT ATC CCT ACG 2496
Thr Gln Leu Lys Val Ile Asn Trp Glu Ser Thr Leu His Ile Pro Thr
820 825 830

CAG GTG AGG CTC AGC GCT GAG GCC CGA GAC CTC ATC ACG AAG CTG TGC 2544
Gln Val Arg Leu Ser Ala Glu Ala Arg Asp Leu Ile Thr Lys Leu Cys
835 840 845

TGC GCG GCT GAC TGC CGC CTG GGC AGG GAT GGG GCA GAT GAC CTC AAG 2592
Cys Ala Ala Asp Cys Arg Leu Gly Arg Asp Gly Ala Asp Asp L u Lys
850 855 860

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GCA CAC CCG TTC TTC AAC ACC ATC GAC TTT TCC CGT GAC ATC CGA AAG      2640
Ala His Pro Phe Phe Asn Thr Ile Asp Phe S r Arg Asp Ile Arg Lys
865                 870                 875                 880

CAG GCT GCA CCC TAC GTC CCC ACC ATC A C CAC CCC ATG GAC ACC TCC      2688
Gln Ala Ala Pro Tyr Val Pro Thr Ile Ser His Pro Met Asp Thr Ser
                885                 890                 895

AAT TTT GAC CCG GTG GAT GAA GAA AGC CCC TGG CAC GAG GCC AGC GGA      2736
Asn Phe Asp Pro Val Asp Glu Glu Ser Pro Trp His Glu Ala Ser Gly
            900                 905                 910

GAG AGC GCC AAG GCC TGG GAC ACG CTG GCC TCC CCC AGC AGC AAG CAT      2784
Glu Ser Ala Lys Ala Trp Asp Thr Leu Ala Ser Pro Ser Ser Lys His
        915                 920                 925

CCA GAG CAC GCC TTC TAT GAG TTC ACC TTC CGC AGG TTC TTC GAT GAC      2832
Pro Glu His Ala Phe Tyr Glu Phe Thr Phe Arg Arg Phe Phe Asp Asp
    930                 935                 940

AAC GGC TAT CCC TTC CGG TGC CCG AAG CCC TCA GAG CCC GCA GAG AGT      2880
Asn Gly Tyr Pro Phe Arg Cys Pro Lys Pro Ser Glu Pro Ala Glu Ser
945                 950                 955                 960

GCA GAC CCA GGG GAT GCG GAC TTG GAA GGT GCG GCC GAG GGC TGC CAG      2928
Ala Asp Pro Gly Asp Ala Asp Leu Glu Gly Ala Ala Glu Gly Cys Gln
                965                 970                 975

CCG GTG TAC GTG TAA GCCTCAGTTA ACCACAACTC GAGGAAACCC AAAATGAGAT      2983
Pro Val Tyr Val  *
            980

TTCTTTTCAG AAGACAAACT CAAGCTTAGG AATCCTTCAT TTTTAGTTCT GGTAAATGGG    3043

CAACAGGAAG AGTCAACATG ATTTCAAATT AGCCCTCTGA GGACCTTCAC TGCATTAAAA    3103

CAGTATTTTT TAAAAAATTA GTACAGTATG GAAAGAGCAC TTATTTTGGG GG            3155
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 981 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Met Arg Ala Thr Pro Lys Phe Gly Pro Tyr Gln Lys Ala Leu Arg Glu
  1               5                  10                  15

Ile Arg Tyr Ser Leu Leu Pro Phe Ala Asn Glu Ser Gly Thr Ser Ala
            20                  25                  30

Ala Ala Glu Val Asn Arg Gln Met Leu Gln Glu Leu Val Asn Ala Ala
        35                  40                  45

Cys Asp Gln Glu Met Ala Gly Arg Ala Leu Thr Gln Thr Gly Ser Arg
    50                  55                  60

Ser Ile Glu Ala Ala Leu Glu Tyr Ile Ser Lys M t Gly Tyr Leu Asp
 65                  70                  75                  80

Pro Arg Asn Glu Gln Il  Val Arg Val Ile Lys Gln Thr Ser Pro Gly
```

85 90 95

Lys Gly Leu Ala Ser Thr Pro Val Thr Arg Arg Pro Ser Phe Glu Gly
100 105 110

Thr Gly Glu Ala Leu Pro Ser Tyr His Gln Leu Gly Gly Ala Asn Tyr
115 120 125

Glu Gly Pro Ala Ala Leu Glu Glu Met Pro Arg Gln Tyr Leu Asp Phe
130 135 140

Leu Phe Pro Gly Ala Gly Ala Gly Thr His Gly Ala Gln Ala His Gln
145 150 155 160

His Pro Pro Lys Gly Tyr Ser Thr Ala Val Glu Pro Ser Ala His Phe
165 170 175

Pro Gly Thr His Tyr Gly Arg Gly His Leu Leu Ser Glu Gln Ser Gly
180 185 190

Tyr Gly Val Gln Arg Ser Ser Ser Phe Gln Asn Lys Thr Pro Pro Asp
195 200 205

Ala Tyr Ser Ser Met Ala Lys Ala Gln Gly Gly Pro Pro Ala Ser Leu
210 215 220

Thr Phe Pro Ala His Ala Gly Leu Tyr Thr Ala Ser His His Lys Pro
225 230 235 240

Ala Ala Thr Pro Pro Gly Ala His Pro Leu His Val Leu Gly Thr Arg
245 250 255

Gly Pro Thr Phe Thr Gly Glu Ser Ser Ala Gln Ala Val Leu Ala Pro
260 265 270

Ser Arg Asn Ser Leu Asn Ala Asp Leu Tyr Glu Leu Gly Ser Thr Val
275 280 285

Pro Trp Ser Ala Ala Pro Leu Ala Arg Arg Asp Ser Leu Gln Lys Gln
290 295 300

Gly Leu Glu Ala Ser Arg Pro His Val Ala Phe Arg Ala Gly Pro Ser
305 310 315 320

Arg Thr Asn Ser Phe Asn Asn Pro Gln Pro Glu Pro Ser Leu Pro Ala
325 330 335

Pro Asn Thr Val Thr Ala Val Thr Ala Ala His Ile Leu His Pro Val
340 345 350

Lys Ser Val Arg Val Leu Arg Pro Glu Pro Gln Thr Ala Val Gly Pro
355 360 365

Ser His Pro Ala Trp Val Ala Ala Pro Thr Ala Pro Ala Thr Glu Ser
370 375 380

Leu Glu Thr Lys Glu Gly Ser Ala Gly Pro His Pro Leu Asp Val Asp
385 390 395 400

Tyr Gly Gly Ser Glu Arg Arg Cys Pro Pro Pro Pro Tyr Pro Lys His
405 410 415

Leu Leu Leu Pro Ser Lys Ser Glu Gln Tyr Ser Val Asp Leu Asp Ser
420 425 430

Leu Cys Thr Ser Val Gln Gln Ser Leu Arg Gly Gly Thr Asp Leu Asp
435 440 445

Gly Ser Asp Lys Ser His Lys Gly Ala Lys Gly Asp Lys Ala Gly Arg
450 455 460

Asp Lys Lys Gln Il Gln Thr S r Pro Val Pr Val Arg Lys Asn Ser
465 470 475 480

Arg Asp Glu Glu Lys Arg Glu Ser Arg Ile Lys Ser Tyr Ser Pro Tyr
485 490 495

Ala Phe Lys Phe Phe Met Glu Gln His Val Glu Asn Val Ile Lys Thr
500 505 510

Tyr Gln Gln Lys Val Ser Arg Arg Leu Gln Leu Glu Gln Glu Met Ala
515 520 525

Lys Ala Gly Leu Cys Glu Ala Glu Gln Glu Gln Met Arg Lys Ile Leu
530 535 540

Tyr Gln Lys Glu Ser Asn Tyr Asn Arg Leu Lys Arg Ala Lys Met Asp
545 550 555 560

Lys Ser Met Phe Val Lys Ile Lys Thr Leu Gly Ile Gly Ala Phe Gly
565 570 575

Glu Val Cys Leu Ala Cys Lys Leu Asp Thr His Ala Leu Tyr Ala Met
580 585 590

Lys Thr Leu Arg Lys Lys Asp Val Leu Asn Arg Asn Gln Val Ala His
595 600 605

Val Lys Ala Glu Arg Asp Ile Leu Ala Glu Ala Asp Asn Glu Trp Val
610 615 620

Val Lys Leu Tyr Tyr Ser Phe Gln Asp Lys Asp Ser Leu Tyr Phe Val
625 630 635 640

Met Asp Tyr Ile Pro Gly Gly Asp Met Met Ser Leu Leu Ile Arg Met
645 650 655

Glu Val Phe Pro Glu His Leu Ala Arg Phe Tyr Ile Ala Glu Leu Thr
660 665 670

Leu Ala Ile Glu Ser Val His Lys Met Gly Phe Ile His Arg Asp Ile
675 680 685

Lys Pro Asp Asn Ile Leu Ile Asp Leu Asp Gly His Ile Lys Leu Thr
690 695 700

Asp Phe Gly Leu Cys Thr Gly Phe Arg Trp Thr His Asn Ser Lys Tyr
705 710 715 720

Tyr Gln Lys Gly Asn His Met Arg Gln Asp Ser Met Glu Pro Gly Asp
725 730 735

Leu Trp Asp Asp Val Ser Asn Cys Arg Cys Gly Asp Arg Leu Lys Thr
740 745 750

Leu Glu Gln Arg Ala Gln Lys Gln His Gln Arg Cys Leu Ala His Ser
755 760 765

Leu Val Gly Thr Pro Asn Tyr Ile Ala Pro Glu Val Leu Leu Arg Lys
770 775 780

Gly Tyr Thr Gln Leu Cys Asp Trp Trp Ser Val Gly Val Ile L u Phe
785 790 795 800

Glu Met Leu Val Gly Gln Pro Pro Phe Leu Ala Pro Thr Pro Thr Glu

805 810 815

Thr Gln Leu Lys Val Ile Asn Trp Glu Ser Thr Leu His Ile Pro Thr
820 825 830

Gln Val Arg Leu Ser Ala Glu Ala Arg Asp Leu Ile Thr Lys Leu Cys
835 840 845

Cys Ala Ala Asp Cys Arg Leu Gly Arg Asp Gly Ala Asp Asp Leu Lys
850 855 860

Ala His Pro Phe Phe Asn Thr Ile Asp Phe Ser Arg Asp Ile Arg Lys
865 870 875 880

Gln Ala Ala Pro Tyr Val Pro Thr Ile Ser His Pro Met Asp Thr Ser
885 890 895

Asn Phe Asp Pro Val Asp Glu Glu Ser Pro Trp His Glu Ala Ser Gly
900 905 910

Glu Ser Ala Lys Ala Trp Asp Thr Leu Ala Ser Pro Ser Ser Lys His
915 920 925

Pro Glu His Ala Phe Tyr Glu Phe Thr Phe Arg Arg Phe Phe Asp Asp
930 935 940

Asn Gly Tyr Pro Phe Arg Cys Pro Lys Pro Ser Glu Pro Ala Glu Ser
945 950 955 960

Ala Asp Pro Gly Asp Ala Asp Leu Glu Gly Ala Ala Glu Gly Cys Gln
965 970 975

Pro Val Tyr Val *
980

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Asp Leu Lys Pro Glu Asn
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 2
(D) OTHER INFORMATION: /label= A
/note= "X at the s cond position can be either Threonin or

Serine."

(ix) FEATURE:
(A) NAME/KEY: Peptid
(B) LOCATION: 5
(D) OTHER INFORMATION: /label= B
/note= "X at the fifth position can either be Tyrosine r Phenylalanine."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Gly Xaa Xaa Xaa Xaa Xaa Ala Pro Glu
1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 620 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Asp Asn Thr Asn Arg Pro His Leu Asn Leu Gly Thr Asn Asp Thr
1 5 10 15

Arg Met Ala Pro Asn Asp Arg Thr Tyr Pro Thr Thr Pro Ser Thr Phe
20 25 30

Pro Gln Pro Val Phe Pro Gly Gln Gln Ala Gly Gly Ser Gln Gln Tyr
35 40 45

Asn Gln Ala Tyr Ala Gln Ser Gly Asn Tyr Tyr Gln Gln Asn His Asn
50 55 60

Asp Pro Asn Thr Gly Leu Ala His Gln Phe Ala His Gln Asn Ile Gly
65 70 75 80

Ser Ala Gly Arg Ala Ser Pro Tyr Gly Ser Arg Gly Pro Ser Pro Ala
85 90 95

Gln Arg Pro Arg Thr Ser Gly Asn Ser Gly Gln Gln Gln Thr Tyr Gly
100 105 110

Asn Tyr Leu Ser Ala Pro Met Pro Ser Asn Thr Gln Thr Glu Phe Ala
115 120 125

Pro Leu Pro Ser Gly Thr Pro Thr Asn Met Ala Pro Met Pro Thr Thr
130 135 140

Thr Arg Arg Ser Ala His Ser Trp Pro Leu Thr Ser Leu Arg Thr Ala
145 150 155 160

Ser Ser Ala Pro Gly Ser Ala Thr Arg Gly Glu Cys Cys Ser Asp Ala
165 170 175

Leu Leu Pro Leu His Pro Ala Val Ile Gly Ala Asp Thr Leu Phe Arg
180 185 190

Gln Ser Glu Met Glu Gln Lys Leu Gly Glu Thr Asn Asp Ala Arg Arg
195 200 205

Arg Glu Ser Ile Trp Ser Thr Ala Gly Arg Lys Glu Gly Gln Tyr Leu
210 215 220

Arg Phe Leu Arg Thr Lys Asp Lys Pro Glu Asn Tyr Gln Thr Ile Lys
225 230 235 240

Ile Ile Gly Lys Gly Ala Phe Gly Glu Val Lys L u Val Gln Lys Lys
245 250 255

Ala Asp Gly Lys Val Tyr Ala Met Lys Ser Leu Ile Lys Thr Glu Met
260 265 270

Phe Lys Lys Asp Gln Leu Ala His Val Arg Ala Glu Arg Asp Ile Leu
275 280 285

Ala Glu Ser Asp Ser Pro Trp Val Val Lys Leu Tyr Thr Thr Phe Gln
290 295 300

Asp Ala Asn Phe Leu Tyr Met Leu Met Glu Phe Leu Pro Gly Gly Asp
305 310 315 320

Leu Met Thr Met Leu Ile Lys Tyr Glu Ile Phe Ser Glu Asp Ile Thr
325 330 335

Arg Phe Tyr Ile Ala Glu Ile Val Leu Ala Ile Asp Ala Val His Lys
340 345 350

Leu Gly Phe Ile His Arg Asp Ile Lys Pro Asp Asn Ile Leu Leu Asp
355 360 365

Arg Gly Gly His Val Lys Leu Thr Asp Phe Gly Leu Ser Thr Gly Phe
370 375 380

His Lys Leu His Asp Asn Asn Tyr Tyr Thr Gln Leu Leu Gln Gly Lys
385 390 395 400

Ser Asn Lys Pro Arg Asp Asn Arg Asn Ser Val Ala Ile Asp Gln Ile
405 410 415

Asn Leu Thr Val Ser Asn Arg Ala Gln Ile Asn Asp Trp Arg Arg Ser
420 425 430

Arg Arg Leu Met Ala Tyr Ser Thr Val Gly Thr Pro Asp Tyr Ile Ala
435 440 445

Pro Glu Ile Phe Thr Gly His Gly Tyr Ser Phe Asp Cys Asp Trp Trp
450 455 460

Ser Leu Gly Thr Ile Met Phe Glu Cys Leu Val Gly Trp Pro Pro Phe
465 470 475 480

Cys Ala Glu Asp Ser His Asp Thr Tyr Arg Lys Ile Val Asn Trp Arg
485 490 495

His Ser Leu Tyr Phe Pro Asp Asp Ile Thr Leu Gly Val Asp Ala Glu
500 505 510

Asn Leu Ile Arg Ser Leu Ile Cys Asn Thr Glu Asn Arg Leu Gly Arg
515 520 525

Gly Gly Ala His Glu Ile Lys Ser His Ala Phe Phe Arg Gly Val Glu
530 535 540

Phe Asp Ser Leu Arg Arg Ile Arg Ala Pro Phe Glu Pro Arg Leu Thr
545 550 555 560

Ser Ala Ile Asp Thr Thr Tyr Phe Pro Thr Asp Glu Il Asp Gln Thr

565 570 575

Asp Asn Ala Thr Leu Leu Lys Ala ln Gln Ala Ala Arg Gly Ala Ala
580 585 590

Ala Pro Ala Gln Gln Glu Glu Ser Pro Glu Leu Ser Leu Pro Phe Ile
595 600 605

Gly Tyr Thr Phe Lys Arg Phe Asp Asn Asn Phe Arg
610 615 620

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 526 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met Asp Ser Ala Arg Gly Trp Phe Gln Lys Leu Ser Ser Thr Lys Lys
1 5 10 15

Asp Pro Met Ala Ser Gly Arg Glu Asp Gly Lys Pro Val Ser Ala Glu
20 25 30

Glu Ala Ser Asn Ile Thr Lys Gln Arg Val Ala Ala Ala Lys Gln Tyr
35 40 45

Ile Glu Lys His Tyr Arg Glu Gln Met Lys Asn Leu Gln Glu Arg Arg
50 55 60

Glu Arg Arg Ile Leu Leu Glu Lys Lys Leu Ala Asp Ala Asp Val Ser
65 70 75 80

Glu Glu Asp Gln Asn Asn Leu Leu Lys Phe Leu Glu Lys Lys Glu Thr
85 90 95

Glu Tyr Met Arg Leu Gln Arg His Lys Met Gly Ala Asp Asp Phe Glu
100 105 110

Leu Leu Thr Met Ile Gly Lys Gly Ala Phe Gly Glu Pro Ile Cys Met
115 120 125

Ile Gly Phe Ser Val Ile Thr Gly Gln Asn Cys Arg Glu Lys Thr Thr
130 135 140

Gly Gln Val Tyr Ala Met Lys Lys Leu Lys Lys Ser Glu Met Leu Arg
145 150 155 160

Arg Gly Gln Val Glu His Val Lys Ala Glu Arg Asn Leu Leu Ala Glu
165 170 175

Val Asp Ser Asp Cys Ile Val Lys Leu Tyr Tyr Ser Phe Gln Asp Asp
180 185 190

Asp Tyr Leu Tyr Leu Val Met Glu Tyr Leu Pro Gly Gly Asp Met Met
195 200 205

Thr Leu Leu Met Arg Lys Asp Ile Leu Thr Glu Asp Glu Ala Arg Phe
210 215 220

```
Tyr Val Ala Glu Thr Val Leu Ala Ile Glu Ser Ile His Lys His Asn
225                 230                 235                 240

Tyr Ile His Arg Asp Ile Lys Pro Asp Asn Leu Leu L u Asp Arg Tyr
                245                 250                 255

Gly His L u Lys Leu Ser Asp Phe Gly Leu Cys Lys Pro Leu Asp Cys
            260                 265                 270

Ser Thr Leu Glu Glu Lys Asp Phe Ser Val Gly Asp Asn Ala Asn Gly
        275                 280                 285

Gly Ser Arg Ser Asp Ser Pro Pro Ala Pro Lys Arg Thr Gln Gln Glu
    290                 295                 300

Gln Leu Glu His Trp Gln Lys Asn Arg Arg Met Leu Ala Tyr Ser Thr
305                 310                 315                 320

Val Gly Thr Pro Asp Tyr Ile Ala Pro Glu Val Leu Leu Lys Lys Gly
                325                 330                 335

Tyr Gly Met Glu Cys Asp Trp Trp Ser Leu Gly Ala Ile Met Tyr Glu
            340                 345                 350

Met Leu Val Gly Tyr Pro Pro Phe Tyr Ser Asp Asp Pro Met Ser Thr
        355                 360                 365

Cys Arg Lys Ile Val Asn Trp Lys Asn His Leu Lys Phe Pro Glu Glu
    370                 375                 380

Ala Lys Leu Ser Pro Glu Ala Lys Asp Ile Ile Ser Arg Leu Leu Cys
385                 390                 395                 400

Asn Val Thr Glu Arg Leu Gly Ser Asn Gly Ala Asp Glu Ile Lys Val
                405                 410                 415

His Ser Trp Phe Lys Gly Ile Asp Trp Asp Arg Ile Tyr Gln Met Glu
            420                 425                 430

Ala Ala Phe Ile Pro Glu Val Asn Asp Glu Leu Asp Thr Gln Asn Phe
        435                 440                 445

Glu Lys Phe Glu Glu Ser Glu Ser His Ser Gln Ser Gly Ser Arg Ser
    450                 455                 460

Gly Pro Trp Arg Lys Met Leu Ser Ser Lys Asp Ile Asn Phe Val Gly
465                 470                 475                 480

Tyr Thr Tyr Lys Asn Phe Lys Val Val Asn Asp Tyr Gln Val Pro Gly
                485                 490                 495

Met Val Glu Leu Lys Lys Thr Asn Thr Lys Pro Lys Lys Pro Thr Ile
            500                 505                 510

Lys Ser Leu Phe Gly Asp Glu Ser Glu Ala Ser Glu Asp Asn
        515                 520                 525
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 479 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Arg Lys Leu His Asp Ala Asp Val S r Glu Glu Asp Gln Asn Asn Leu
1 5 10 15

Leu Lys Phe Leu Glu Lys Lys Glu Thr Glu Tyr Met Arg L u Gln Arg
20 25 30

His Lys Met Gly Ala Asp Asp Phe Glu Leu Leu Thr Met Ile Gly Lys
35 40 45

Gly Ala Phe Gly Glu Val Arg Val Cys Arg Glu Lys Thr Thr Gly His
50 55 60

Val Tyr Ala Met Lys Lys Leu Lys Lys Ser Glu Met Leu Arg Arg Gly
65 70 75 80

Gln Val Glu His Val Lys Ala Glu Arg Asn Leu Leu Ala Glu Val Asp
85 90 95

Ser Asn Cys Ile Val Lys Leu Tyr Cys Ser Phe Gln Asp Glu Glu Tyr
100 105 110

Leu Tyr Leu Ile Met Glu Tyr Leu Pro Gly Gly Asp Met Met Thr Leu
115 120 125

Leu Met Arg Lys Asp Thr Leu Thr Glu Asp Glu Ala Arg Phe Tyr Val
130 135 140

Ala Glu Thr Ile Leu Ala Ile Glu Ser Ile His Lys His Asn Tyr Ile
145 150 155 160

His Arg Asp Ile Lys Pro Asp Asn Leu Leu Leu Asp Lys Phe Gly His
165 170 175

Leu Arg Leu Ser Asp Phe Gly Leu Cys Lys Pro Leu Asp Cys Ser Thr
180 185 190

Leu Glu Glu Lys Asp Phe Glu Val Asn Asn Gly Asn Gly Gly Ser Pro
195 200 205

Ser Asn Glu Gly Ser Thr Lys Pro Arg Arg Thr Gln Gln Glu Gln Leu
210 215 220

Gln His Trp Gln Lys Asn Arg Arg Met Leu Ala Tyr Ser Thr Val Gly
225 230 235 240

Thr Pro Asp Tyr Ile Ala Pro Glu Val Leu Leu Lys Lys Gly Tyr Gly
245 250 255

Met Glu Cys Asp Trp Trp Ser Leu Gly Ala Ile Met Tyr Glu Met Leu
260 265 270

Val Gly Tyr Pro Pro Phe Tyr Ser Asp Asp Pro Met Ser Thr Cys Arg
275 280 285

Lys Ile Val Asn Trp Arg Thr His Leu Lys Phe Pro Glu Glu Ala Lys
290 295 300

Leu Ser Pro Glu Ala Lys Asp Leu Ile Ser Lys Leu Leu Cys Asn Val
305 310 315 320

Thr Gln Arg Leu Gly Ser Asn Gly Ala His Glu Ile Lys Leu His Pro
325 330 335

Trp Phe Asn Gly Ile Asp Trp Glu Arg Ile Tyr Gln Met Glu Ala Ala

340 345 350

Ph Ile Pro Glu Val Asn Asp Glu Leu Asp Thr Gln Asn Phe Glu Lys
355 360 365

Phe Glu Glu Ala Asp Asn Ser Ser Gln Ser Thr Ser Lys Ala Gly Pro
370 375 380

Trp Arg Lys Met Leu Ser Ser Lys Asp Leu Asn Phe Val Gly Tyr Thr
385 390 395 400

Tyr Lys Asn Phe Glu Ile Val Asn Asp Tyr Gln Val Pro Gly Ile Ala
405 410 415

Glu Leu Lys Lys Lys Asp Thr Lys Pro Lys Arg Pro Ser Ile Lys Ser
420 425 430

Leu Phe Glu Asp Glu Ser Ser Asp Ser Ser Glu Ala Ala Thr Ser Gly
435 440 445

Asp Gln Ser Val Gln Gly Ser Phe Leu Asn Leu Leu Pro Pro Gln Leu
450 455 460

Glu Val Ser Gln Thr Gln Thr Glu Val Pro Pro Pro Lys Phe Thr
465 470 475

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 500 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Met Glu Lys Val Lys Ala Ala Lys Lys Phe Ile Glu Asn His Tyr Arg
1 5 10 15

Ser Gln Met Lys Asn Ile Gln Glu Arg Lys Glu Arg Arg Trp Val Leu
20 25 30

Glu Lys Gln Leu Ala Ser Ser Asp Val Pro Glu Glu Glu Gln Met Ser
35 40 45

Leu Ile Lys Asp Leu Glu Arg Lys Glu Thr Glu Phe Met Arg Leu Lys
50 55 60

Arg Asn Arg Ile Cys Val Asn Asp Phe Glu Leu Leu Thr Ile Ile Gly
65 70 75 80

Arg Gly Ala Tyr Gly Glu Val Gln Leu Cys Arg Glu Lys Lys Ser Glu
85 90 95

Asn Ile Tyr Ala Met Lys Lys Leu Lys Lys Ser Glu Met Leu Ser Arg
100 105 110

Gly Gln Val Glu His Val Arg Ala Glu Arg Asn Leu Leu Ala Glu Val
115 120 125

Asp Ser His Cys Ile Val Lys Leu Phe Tyr Ser Phe Gln Asp Ala Glu
130 135 140

Tyr Leu Tyr Leu Ile Met Glu Tyr L u Pro Gly Gly Asp M t Met Thr
145 150 155 160

Leu Leu Met Arg Glu Asp Ile Leu Thr Glu Lys Val Ala Lys Phe Tyr
165 170 175

Ile Ala Gln Ser Val Leu Ala Ile Glu Ser Ile His Lys His Asn Tyr
180 185 190

Ile His Arg Asp Ile Lys Pro Asp Asn Leu Leu Leu Asp Lys Asn Gly
195 200 205

His Met Lys Leu Ser Asp Phe Gly Leu Cys Lys Pro Leu Asp Cys Ala
210 215 220

Thr Leu Ser Thr Ile Lys Glu Asn Glu Ser Met Asp Asp Val Ser Lys
225 230 235 240

Asn Ser Met Asp Ile Asp Ala Ser Leu Pro Asp Ala Gly Asn Gly His
245 250 255

Ser Trp Arg Ser Ala Arg Glu Gln Leu Gln His Trp Gln Arg Asn Arg
260 265 270

Arg Lys Leu Ala Phe Ser Thr Val Gly Thr Pro Asp Tyr Ile Ala Pro
275 280 285

Glu Val Leu Leu Lys Lys Gly Tyr Gly Met Glu Cys Asp Trp Trp Ser
290 295 300

Leu Gly Ala Ile Met Tyr Glu Met Leu Val Gly Tyr Pro Pro Phe Tyr
305 310 315 320

Ser Asp Asp Pro Ile Thr Thr Cys Arg Lys Ile Val His Trp Arg His
325 330 335

Tyr Leu Lys Phe Pro Asp Asp Ala Lys Leu Thr Phe Glu Ala Arg Asp
340 345 350

Leu Ile Cys Arg Leu Leu Cys Asp Val Glu His Arg Leu Gly Thr Gly
355 360 365

Gly Ala Glu Gln Ile Lys Val His Ala Trp Phe Lys Asp Val Glu Trp
370 375 380

Asp Arg Leu Tyr Glu Thr Asp Ala Ala Tyr Lys Pro Gln Val Asn Gly
385 390 395 400

Glu Leu Asp Thr Gln Asn Phe Met Lys Phe Asp Glu Ala Asn Pro Pro
405 410 415

Thr Pro Ser Arg Ser Gly Ser Gly Pro Ser Arg Lys Met Leu Thr Ser
420 425 430

Lys Asp Leu Ser Phe Val Gly Tyr Thr Tyr Lys Asn Phe Asp Ala Val
435 440 445

Lys Gly Leu Lys His Ser Phe Asp Arg Lys Gly Ser Thr Ser Pro Lys
450 455 460

Arg Pro Ser Leu Asp Ser Met Phe Asn Glu Asn Gly Met Asp Tyr Thr
465 470 475 480

Ala Lys His Ala Glu Glu Met Asp Val Gln Met Leu Thr Ala Asp Asp
485 490 495

Cys Met Ser Pro

500

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 564 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Met Phe Ser Arg Ser Asp Arg Glu Val Asp Asp Leu Ala Gly Asn Met
1 5 10 15

Ser His Leu Gly Phe Tyr Asp Leu Asn Ile Pro Lys Pro Thr Ser Pro
20 25 30

Gln Ala Gln Tyr Arg Pro Ala Arg Lys Ser Glu Asn Gly Arg Leu Thr
35 40 45

Pro Gly Leu Pro Arg Ser Tyr Lys Pro Cys Asp Ser Asp Asp Gln Asp
50 55 60

Thr Phe Lys Asn Arg Ile Ser Leu Asn His Ser Pro Lys Lys Leu Pro
65 70 75 80

Lys Asp Phe His Glu Arg Ala Ser Gln Ser Lys Thr Gln Arg Val Val
85 90 95

Asn Val Cys Gln Leu Tyr Phe Leu Asp Tyr Tyr Cys Asp Met Phe Asp
100 105 110

Tyr Val Ile Ser Arg Arg Gln Arg Thr Lys Gln Val Leu Arg Tyr Leu
115 120 125

Glu Gln Gln Arg Ser Val Lys Asn Val Ser Asn Lys Val Leu Asn Glu
130 135 140

Glu Trp Ala Leu Tyr Leu Gln Arg Glu His Glu Val Leu Arg Lys Arg
145 150 155 160

Arg Leu Lys Pro Lys His Lys Asp Phe Gln Ile Leu Thr Gln Val Gly
165 170 175

Gln Gly Gly Tyr Gly Gln Val Tyr Leu Ala Lys Lys Lys Asp Ser Asp
180 185 190

Glu Ile Cys Ala Leu Lys Ile Leu Asn Lys Lys Leu Leu Phe Lys Leu
195 200 205

Asn Glu Thr Asn His Val Leu Thr Glu Arg Asp Ile Leu Thr Thr Thr
210 215 220

Arg Ser Asp Trp Leu Val Lys Leu Leu Tyr Ala Phe Gln Asp Pro Glu
225 230 235 240

Ser Leu Tyr Leu Ala Met Glu Phe Val Pro Gly Gly Asp Phe Arg Thr
245 250 255

L u Leu Ile Asn Thr Arg Ile Leu Lys Ser Gly His Ala Arg Ph Tyr
260 265 270

Il Ser Glu Met Phe Cys Ala Val Asn Ala Leu His Glu Leu Gly Tyr
275 280 285

Thr His Arg Asp Leu Lys Pro Glu Asn Ph L u Ile Asp Ala Thr Gly
290 295 300

His Ile Lys Leu Thr Asp Phe Gly Leu Ala Ala Gly Thr Val S r Asn
305 310 315 320

Glu Arg Ile Glu Ser Met Lys Ile Arg Leu Glu Glu Val Lys Asn Leu
325 330 335

Gln Phe Pro Ala Phe Thr Glu Arg Ser Ile Glu Asp Arg Ser Lys Ile
340 345 350

Tyr His Asn Met Arg Lys Thr Glu Ile Asn Tyr Ala Asn Ser Met Val
355 360 365

Gly Ser Pro Asp Tyr Met Ala Leu Glu Val Leu Glu Gly Lys Lys Tyr
370 375 380

Asp Phe Thr Val Asp Tyr Trp Ser Leu Gly Cys Met Leu Phe Glu Ser
385 390 395 400

Leu Val Gly Tyr Thr Pro Phe Ser Gly Ser Ser Thr Asn Glu Thr Tyr
405 410 415

Glu Asn Leu Arg Tyr Trp Lys Lys Thr Leu Arg Arg Pro Arg Thr Glu
420 425 430

Asp Arg Arg Ala Ala Phe Ser Asp Arg Thr Trp Asp Leu Ile Thr Arg
435 440 445

Leu Ile Ala Asp Pro Ile Asn Arg Val Arg Ser Phe Glu Gln Val Arg
450 455 460

Lys Met Ser Tyr Phe Ala Glu Ile Asn Phe Glu Thr Leu Arg Thr Ser
465 470 475 480

Ser Pro Pro Phe Ile Pro Gln Leu Asp Asp Glu Thr Asp Ala Gly Tyr
485 490 495

Phe Asp Asp Phe Thr Asn Glu Glu Asp Met Ala Lys Tyr Ala Asp Val
500 505 510

Phe Lys Arg Gln Asn Lys Leu Ser Ala Met Val Asp Asp Ser Ala Val
515 520 525

Asp Ser Lys Leu Val Gly Phe Thr Phe Arg His Arg Asp Gly Lys Gln
530 535 540

Gly Ser Ser Gly Ile Leu Tyr Asn Gly Ser Glu His Ser Asp Pro Phe
545 550 555 560

Ser Thr Phe Tyr

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 561 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID N :16:

Met Ala Gly Asn Met Ser Asn Leu Ser Phe Asp Gly His Gly Thr Pro
1 5 10 15

Gly Gly Thr Gly Leu Phe Pro Asn Gln Asn Il Thr Lys Arg Arg Thr
20 25 30

Arg Pro Ala Gly Ile Asn Asp Ser Pro Ser Pro Val Lys Pro Ser Phe
35 40 45

Phe Pro Tyr Glu Asp Thr Ser Asn Met Asp Ile Asp Glu Val Ser Gln
50 55 60

Pro Asp Met Asp Val Ser Asn Ser Pro Lys Lys Leu Pro Pro Lys Phe
65 70 75 80

Tyr Glu Arg Ala Thr Ser Asn Lys Thr Gln Arg Val Val Ser Val Cys
85 90 95

Lys Met Tyr Phe Leu Glu Tyr Tyr Cys Asp Met Phe Asp Tyr Val Ile
100 105 110

Ser Arg Arg Gln Arg Thr Lys Gln Val Leu Glu Tyr Leu Gln Gln Gln
115 120 125

Ser Gln Leu Pro Asn Ser Asp Gln Ile Lys Leu Asn Glu Glu Trp Ser
130 135 140

Ser Tyr Leu Gln Arg Glu His Gln Val Leu Arg Lys Arg Arg Leu Lys
145 150 155 160

Pro Lys Asn Arg Asp Phe Glu Met Ile Thr Gln Val Gly Gln Gly Gly
165 170 175

Tyr Gly Gln Val Tyr Leu Ala Arg Lys Lys Asp Thr Lys Glu Val Cys
180 185 190

Ala Leu Lys Ile Leu Asn Lys Lys Leu Leu Phe Lys Leu Asn Glu Thr
195 200 205

Lys His Val Leu Thr Glu Arg Asp Ile Leu Thr Thr Thr Arg Ser Glu
210 215 220

Trp Leu Val Lys Leu Leu Tyr Ala Phe Gln Glu Leu Gln Ser Leu Tyr
225 230 235 240

Leu Ala Met Glu Phe Val Pro Gly Gly Asp Phe Arg Thr Leu Leu Ile
245 250 255

Asn Thr Arg Cys Leu Lys Ser Gly His Ala Arg Phe Tyr Ile Ser Glu
260 265 270

Met Phe Cys Ala Val Asn Ala Leu His Asp Leu Gly Tyr Thr His Arg
275 280 285

Asp Leu Lys Pro Glu Asn Phe Leu Ile Asp Ala Lys Gly His Ile Lys
290 295 300

Leu Thr Asp Phe Gly Leu Ala Ala Gly Thr Ile Ser Asn Glu Arg Ile
305 310 315 320

Glu Ser Met Lys Ile Arg Leu Glu Lys Ile Lys Asp Leu Glu Phe Pro
325 330 335

Ala Phe Thr Glu Lys Ser Ile Glu Asp Arg Arg Lys Met Tyr Asn Gln

340 345 350

Leu Arg Glu Lys Glu Ile Asn Tyr Ala Asn Ser Met Val Gly Ser Pro
355 360 365

Asp Tyr M t Ala Leu Glu Val Leu Glu Gly Lys Lys Tyr Asp Phe Thr
370 375 380

Val Asp Tyr Trp Ser Leu Gly Cys Met Leu Phe Glu Ser Leu Val Gly
385 390 395 400

Tyr Thr Pro Phe Ser Gly Ser Ser Thr Asn Glu Thr Tyr Asp Asn Leu
405 410 415

Arg Arg Trp Lys Gln Thr Leu Arg Arg Pro Arg Gln Ser Asp Gly Arg
420 425 430

Ala Ala Phe Ser Asp Arg Thr Trp Asp Leu Ile Thr Arg Leu Ile Ala
435 440 445

Asp Pro Ile Asn Arg Leu Arg Ser Phe Glu His Val Lys Arg Met Ser
450 455 460

Tyr Phe Ala Asp Ile Asn Phe Ser Thr Leu Arg Ser Met Ile Pro Pro
465 470 475 480

Phe Thr Pro Gln Leu Asp Ser Glu Thr Asp Ala Gly Tyr Phe Asp Asp
485 490 495

Phe Thr Ser Glu Ala Asp Met Ala Lys Tyr Ala Asp Val Phe Lys Arg
500 505 510

Gln Asp Lys Leu Thr Ala Met Val Asp Asp Ser Ala Val Ser Ser Lys
515 520 525

Leu Val Gly Phe Thr Phe Arg His Arg Asn Gly Lys Gln Gly Ser Ser
530 535 540

Gly Ile Leu Phe Asn Gly Leu Glu His Ser Asp Pro Phe Ser Thr Phe
545 550 555 560

Tyr

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 108, lines 1-20 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet

Name of depositary institution

American Type Culture Collection

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, MD 20852
US

Date of deposit March 24, 1995 Accession Number 69769

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☑ This sheet was received with the International application when filed (to be checked by the receiving Office)

Catherine Williams
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

WHAT IS CLAIMED IS:

1. A purifi d lats protein.

2. The protein of claim 1 which is a human protein.

3. The protein of claim 1 which is a *D. melanogaster* protein.

4. The protein of claim 1 which is a mouse protein.

5. The protein of claim 1 which is a mammalian protein.

6. The protein of claim 2 which comprises the amino acid sequence substantially as set forth in SEQ ID NO:4.

7. A purified protein encoded by a nucleic acid hybridizable to the lats DNA sequence in plasmid PBS(KS)-h-lats as deposited with the ATCC and assigned accession number 69769.

8. A purified protein encoded by a nucleic acid hybridizable to a DNA having a sequence consisting of the coding region of SEQ ID NO:7.

9. The protein of claim 2 which is encoded by plasmid pBS(KS)-h-lats as deposited with the ATCC and assigned accession number 69769.

10. A purified derivative or analog of the protein of claim 1, which displays one or more functional activities of a lats protein.

11. The derivative or analog of claim 10 which is able to be bound by an antibody directed against a lats protein.

12. A purified fragment of a lats protein comprising a domain of the lats protein selected from the group consisting of a lats C-terminal domain 3 (LCD3), lats C-terminal domain 2 (LCD2), lats C-terminal domain 1 (LCD1), kinase domain, a kinase subdomain, lats flanking domain (LFD), lats split domain 1 (LSD1), lats split domain 2 (LSD2), SH3-binding domain, and opa repeat domain.

13. A molecule comprising the fragment of claim 12.

14. A protein comprising an amino acid sequence that has at least 60% identity to a domain of a lats protein, in which the percentage identity is determined over an amino acid sequence of identical size to the domain.

15. A protein comprising an amino acid sequence that has at least 90% identity to a domain of a lats protein, in which the percentage identity is determined over an amino acid sequence of identical size to the domain.

16. The derivative or analog of claim 10, which inhibits proliferation of a cell.

17. A chimeric protein comprising a fragment of a lats protein consisting of at least 6 amino acids fused via a covalent bond to an amino acid sequence of a second protein, in which the second protein is not a lats protein.

18. The chimeric protein of claim 17 in which the fragment of a lats protein is a fragment capable of being bound by an anti-lats antibody.

19. The fragment of claim 12 which additionally lacks one or more domains of the lats protein.

20. An antibody which is capable of binding a lats protein.

21. The antibody of claim 20 which is monoclonal.

22. A molecule comprising a fragment of the antibody of claim 21, which fragment is capable of binding a lats protein.

23. An isolated nucleic acid comprising a nucleotide sequence encoding a lats protein.

24. The nucleic acid of claim 23 which is a DNA.

25. An isolated nucleic acid comprising a nucleotide sequence complementary to the nucleotide sequence of claim 23.

26. The nucleic acid of claim 23 in which the lats protein is a human lats protein.

27. An isolated nucleic acid comprising the *lats* coding sequence contained in plasmid pBS(KS)-h-lats as deposited with the ATCC and assigned accession number 69769.

28. An isolated nucleic acid hybridizable to the *lats* DNA sequence in plasmid pBS(KS)-h-lats as deposited with the ATCC and assigned accession number 69769.

29. An isolated nucleic acid hybridizable to a DNA having a sequence consisting of the coding region of SEQ ID NO:7.

30. An isolated nucleic acid comprising a fragment of a *lats* g ne consisting of at least 8 nucleotid s.

31. An is lated nucleic acid comprising a nucleotide sequence encoding a fragment of a lats protein that displays one or more functional activities of the lats protein.

32. An isolated nucleic acid comprising a nucleotide sequence encoding the chimeric protein of claim 17.

33. An isolated nucleic acid comprising a nucleotide sequence encoding a protein, said protein comprising the amino acid sequence of SEQ ID NO:4.

34. An isolated nucleic acid comprising a nucleotide sequence encoding the fragment of claim 12.

35. An isolated nucleic acid comprising a nucleotide sequence encoding the protein of claim 14.

36. A recombinant cell containing the nucleic acid of claim 23, in which the nucleotide sequence encoding the lats protein is under the control of a promoter that is not a native *lats* gene promoter.

37. A recombinant cell containing a nucleic acid vector comprising the nucleic acid of claim 26.

38. A recombinant cell containing the nucleic acid of claim 34.

39. A recombinant cell containing the nucleic acid of claim 35.

40. A method of producing a lats protein comprising growing a recombinant cell containing the nucleic acid of claim 23, in which th nucleotide sequence encoding the lats protein is und r the control of a promoter that is not a native *lats* gene promoter, such that the encoded lats protein is expressed by the cell, and recovering the expressed lats protein.

41. A method of producing a lats protein comprising growing a recombinant cell containing a nucleic acid vector comprising the nucleic acid of claim 26 such that the encoded lats protein is expressed by the cell, and recovering the expressed lats protein.

42. A method of producing a lats fragment comprising growing a recombinant cell containing the nucleic acid of claim 34 such that the encoded lats fragment is expressed by the cell, and recovering the expressed lats fragment.

43. A method of producing a protein comprising a fragment of a lats protein, which method comprises growing a recombinant cell containing the nucleic acid of claim 35 such that the encoded protein is expressed by the cell, and recovering the expressed protein.

44. The product of the process of claim 40.

45. The product of the process of claim 41.

46. The product of the process of claim 42.

47. The product of the process of claim 43.

48. A pharmaceutical composition comprising a therapeutically effective amount of a lats protein; and a pharmaceutically acceptable carrier.

49. The composition of claim 48 in which the lats protein is a human lats protein.

50. A pharmaceutical composition comprising a therapeutically effective amount of the fragment of claim 12; and a pharmaceutically acceptable carrier.

51. A pharmaceutical composition comprising a therapeutically effective amount of the protein of claim 14; and a pharmaceutically acceptable carrier.

52. A pharmaceutical composition comprising a therapeutically effective amount of the chimeric protein of claim 17; and a pharmaceutically acceptable carrier.

53. A pharmaceutical composition comprising a therapeutically effective amount of the nucleic acid of claim 23; and a pharmaceutically acceptable carrier.

54. A pharmaceutical composition comprising a therapeutically effective amount of the nucleic acid of claim 35; and a pharmaceutically acceptable carrier.

55. A pharmaceutical composition comprising a therapeutically effective amount of the recombinant cell of claim 36; and a pharmaceutically acceptable carrier.

56. A pharmaceutical composition comprising a therapeutically effective amount of an antibody that immunospecifically binds to a lats protein; and a pharmaceutically acceptable carrier.

57. A pharmaceutical composition comprising a therapeutically effective amount of a fragment or derivative of an antibody that immunospecifically binds to a lats protein, said fragment or derivative containing the binding

domain of the antibody; and a pharmaceutically acceptable carrier.

58. A method of treating or prev nting a disease or disorder involving cell overproliferation in a subject comprising administering to a subject in which such treatment or prevention is desired a therapeutically effective amount of a molecule that promotes lats function.

59. The method according to claim 58 in which the disease or disorder is a malignancy.

60. The method according to claim 59 in which the disease or disorder is selected from the group consisting of bladder cancer, breast cancer, colon cancer, leukemia, lung cancer, melanoma, pancreatic cancer, sarcoma, and uterine cancer.

61. The method according to claim 58 in which the subject is a human.

62. The method according to claim 58 in which the disease or disorder is selected from the group consisting of premalignant conditions, benign tumors, hyperproliferative disorders, and benign dysproliferative disorders.

63. The method according to claim 58 in which the molecule that promotes lats function is selected from the group consisting of a lats protein, a lats derivative or analog that is active in inhibiting cell proliferation, a nucleic acid encoding a lats protein, and a nucleic acid encoding a lats derivative or analog that is active in inhibiting cell proliferation.

64. The method according to claim 58 in which the molecule that promotes lats function is a lats derivative or

anal g that comprises a kinase domain of a lats protein that has b n mutated so as to be dominantly active.

65. Th method according to claim 58 in which the molecule that promotes lats function is the protein of claim 14.

66. A method of treating or preventing a disease or disorder involving a deficiency in cell proliferation or in which cell proliferation is desirable for treatment or prevention in a subject comprising administering to a subject in which such treatment or prevention is desired a therapeutically effective amount of a molecule that inhibits lats function.

67. The method according to claim 66 in which the molecule that inhibits lats function is selected from the group consisting of an anti-lats antibody or a fragment or derivative thereof containing the binding region thereof, a lats derivative or analog that is capable of being bound by an anti-lats antibody and that is a dominant-negative protein kinase, a *lats* antisense nucleic acid, and a nucleic acid comprising at least a portion of a *lats* gene into which a heterologous nucleotide sequence has been inserted such that said heterologous sequence inactivates the biological activity of the at least a portion of the *lats* gene, in which the *lats* gene portion flanks the heterologous sequence so as to promote homologous recombination with a genomic *lats* gene.

68. The method according to claim 66 in which the molecule that inhibits lats function is an oligonucleotide which (a) consists of at least six nucleotides; (b) comprises a sequence complementary to at least a portion of an RNA transcript of a *lats* gene; and (c) is hybridizable to the RNA transcript under moderately stringent conditions.

69. The method according to claim 66 in which the dis as or disorder is s l cted from the group consisting of d g nerative disorders, growth deficiencies, hypoproliferativ disorders, physical trauma, lesions, and wounds.

70. An isolated oligonucleotide consisting of at least six nucleotides, and comprising a sequence complementary to at least a portion of an RNA transcript of a *lats* gene, which oligonucleotide is hybridizable to the RNA transcript under moderately stringent conditions.

71. A pharmaceutical composition comprising the oligonucleotide of claim 70; and a pharmaceutically acceptable carrier.

72. A method of inhibiting the expression of a nucleic acid sequence encoding a lats protein in a cell comprising providing the cell with an effective amount of the oligonucleotide of claim 70.

73. A method of diagnosing a disease or disorder characterized by an aberrant level of lats RNA or protein in a subject, comprising measuring the level of lats RNA or protein in a sample derived from the subject, in which an increase or decrease in the level of lats RNA or protein, relative to the level of lats RNA or protein found in an analogous sample not having the disease or disorder indicates the presence of the disease or disorder in the subject.

74. A method of diagnosing or screening for the presence of or a predisposition for developing a disease or disorder involving cell overproliferation in a subject comprising measuring the level of lats protein, lats RNA or lats functional activity in a sample derived from the subject, in which a decrease in the level of lats protein,

lats RNA, r lats functional activity in the sample, relative to the level of lats protein, lats RNA, or lats functional activity found in an analogous sample not having the disease or disorder or a predisposition for developing the disease or disorder, indicates the presence of the disease or disorder or a predisposition for developing the disease or disorder.

75. A method of diagnosing or screening for the presence of or a predisposition for developing a disease or disorder involving cell overproliferation in a subject comprising detecting one or more mutations in lats DNA, RNA or protein derived from the subject in which the presence of said one or more mutations indicates the presence of the disease or disorder or a predisposition for developing the disease or disorder.

76. A method of diagnosing or screening for the presence of or a predisposition for developing a disease or disorder involving a deficiency in cell proliferation or in which cell proliferation is desirable for treatment or prevention in a subject comprising measuring the level of lats protein, lats RNA or lats functional activity in a sample derived from the subject, in which an increase in the level of lats protein, lats RNA, or lats functional activity in the sample, relative to the level of lats protein, lats RNA, or lats functional activity found in an analogous sample not having the disease or disorder or a predisposition for developing the disease or disorder, indicates the presence of the disease or disorder or a predisposition for developing the disease or disorder.

77. A kit comprising in one or more containers a molecule selected from the group consisting of an anti-lats antibody, a nucleic acid probe capable of hybridizing to a *lats* RNA, or a pair of nucleic acid primers capable of priming amplification of at 1 ast a portion of a *lats* nucleic acid.

78. A kit comprising in a container a th rapeutically effective amount of a lats protein.

79. A m thod f increasing cell growth in animals or plants comprising inhibiting lats expression or activity in said animals or plants.

80. The method of claim 79 in which cell growth is increased in an edible plant.

81. The method of claim 79 in which cell growth is increased in a farm animal.

82. A method of identifying a molecule that specifically binds to a ligand selected from the group consisting of a lats protein, a fragment of a lats protein comprising a domain of the protein, and a nucleic acid encoding the protein or fragment, comprising

(a) contacting said ligand with a plurality of molecules under conditions conducive to binding between said ligand and the molecules; and

(b) identifying a molecule within said plurality that specifically binds to said ligand.

83. A recombinant non-human animal or plant that is the product of a process comprising introducing a nucleic acid encoding at least a domain of a lats protein into the plant or animal.

84. A recombinant plant containing and capable of expressing a *lats* antisense nucleic acid.

85. A recombinant non-human animal or plant in which a *lats* gene has been inactivated by a method comprising introducing a nucleic acid into the plant or animal or an ancestor thereof, which nucleic acid comprises a non-*lats*

s quence flanked by *lats* genomic sequences that promote homologous r combination.

86. A method of identifying a tumor suppressor gene comprising (a) identifying an overproliferation phenotype in a genetic mosaic; and (b) isolating a gene that is mutated in cells exhibiting said overproliferation phenotype.

87. The method of claim 86 in which the genetic mosaic is an animal containing (a) a nucleic acid encoding and capable of expressing a recombinase, and (b) intrachromosomal insertions of a target site at which the recombinase promotes recombination, on the homologous arms of both of a set of parental chromosomes; and the genetic mosaic has been produced by a method comprising inducing expression of the recombinase.

88. The method of claim 87 in which the recombinase is an FLP recombinase, and the target site is an FRT site.

89. The method according to claim 87 in which the recombinase is a Cre recombinase, and the target site is a lox site.

90. The method of claim 86 in which the overproliferation phenotype is the formation of overproliferated outgrowth tissue.

91. The method of claim 86 in which the overproliferation phenotype is the formation of a normal structure of larger than normal size.

92. A non-human mammal comprising (a) a nucleic acid sequence encoding a recombinase operably linked to a promoter; and (b) intrachromosomal insertions into the

homologous arms of both of a s t of parental chromosomes, of a target site at which the recombinase can promote r combination.

93. The mammal of claim 92 which is heterozygous for an induced mutation.

94. The mammal of claim 93 in which the sequence encoding the recombinase is operably linked to an inducible promoter.

95. A method of making a genetic mosaic comprising inducing expression of the recombinase in the mammal of claim 93.

96. A method for identifying a gene with an identifiable mutant phenotype comprising:

(a) identifying a mutant phenotype in a genetic mosaic animal, said genetic mosaic animal having been produced by a method comprising recombinantly expressing a recombinase within a cell of the animal so as to promote recombination at intrachromosomally inserted target sites on the homologous arms of both of a set of parental chromosomes; and

(b) isolating a gene that is mutated in cells exhibiting said mutant phenotype.

97. A method for identifying a gene with an identifiable mutant phenotype comprising:

(a) identifying a mutant phenotype in a cultured cell, said cultured cell having been produced by a method comprising recombinantly expressing a recombinase within said cultured cell so as to promote recombination at intrachromosomally inserted target sit s on

the homologous arms of both of a set of parental chromosomes; and

(b) isolating a gene that is mutated in cells exhibiting said mutant phenotype.

98. The method of claim 97 in which the mutant phenotype is a transformed phenotype.

99. The mammal of claim 92 in which the promoter is not a native recombinase gene promoter.

100. A method of inhibiting cellular senescence in a subject comprising administering to a subject in which such inhibition is desired an amount of a molecule that inhibits lats function, effective to inhibit cellular senescence.

101. A method of inhibiting cellular senescence in cells *in vitro* comprising contacting cells *in vitro* with an amount of a molecule that inhibits lats function, effective to inhibit cellular senescence.

102. The method according to claim 100 in which the molecule that inhibits lats function is selected from the group consisting of an anti-lats antibody or a fragment or derivative thereof containing the binding region thereof, a lats derivative or analog that is capable of being bound by an anti-lats antibody and that is a dominant-negative protein kinase, a *lats* antisense nucleic acid, and a nucleic acid comprising at least a portion of a *lats* gene into which a heterologous nucleotide sequence has been inserted such that said heterologous sequence inactivates the biological activity of the at least a portion of the *lats* gene, in which the *lats* gene portion flanks the heterologous sequence so as to promote homologous recombination with a genomic *lats* gene.

103. The method according to claim 101 in which the molecule that inhibits lats function is selected from the

gr up consisting of an anti-lats antibody or a fragment or derivative thereof containing the binding region thereof, a lats derivative or analog that is capable of being bound by an anti-lats antibody and that is a dominant-negative protein kinase, a *lats* antisense nucleic acid, and a nucleic acid comprising at least a portion of a *lats* gene into which heterologous nucleotide sequence has been inserted such that said heterologous sequence inactivates the biological activity of the at least a portion of the *lats* gene, in which the *lats* gene portion flanks the heterologous sequence so as to promote homologous recombination with a genomic *lats* gene.

BAD ORIGINAL

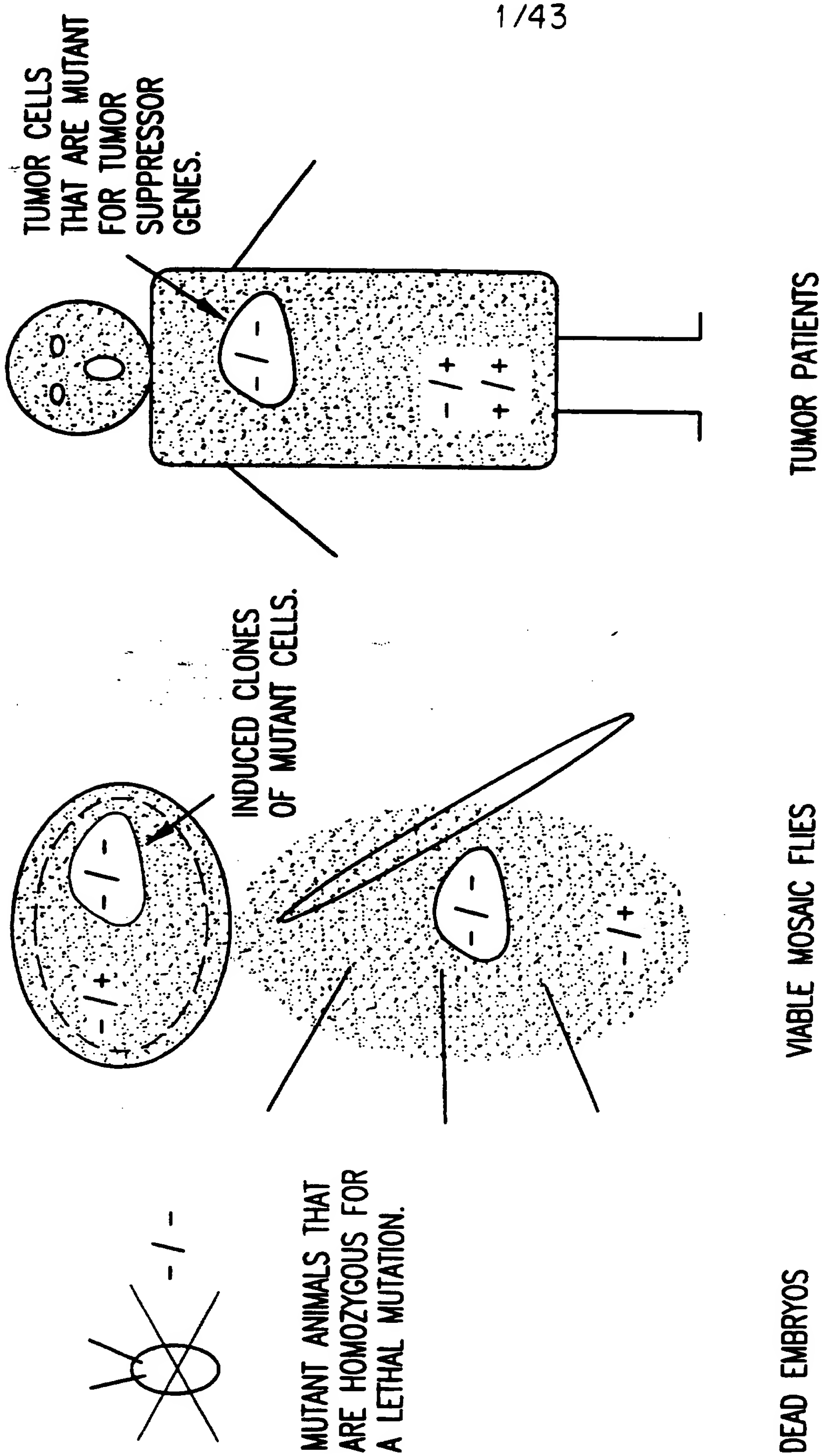
TUMOR CELLS THAT ARE MUTANT FOR TUMOR SUPPRESSOR GENES.
-/-
-/+
+/+
TUMOR PATIENTS
INDUCED CLONES OF MUTANT CELLS.
-/-
-/+
-/-
-/+
VIABLE MOSAIC FLIES
-/-
MUTANT ANIMALS THAT ARE HOMOZYGOUS FOR A LETHAL MUTATION.
DEAD EMBRYOS

FIG.1A

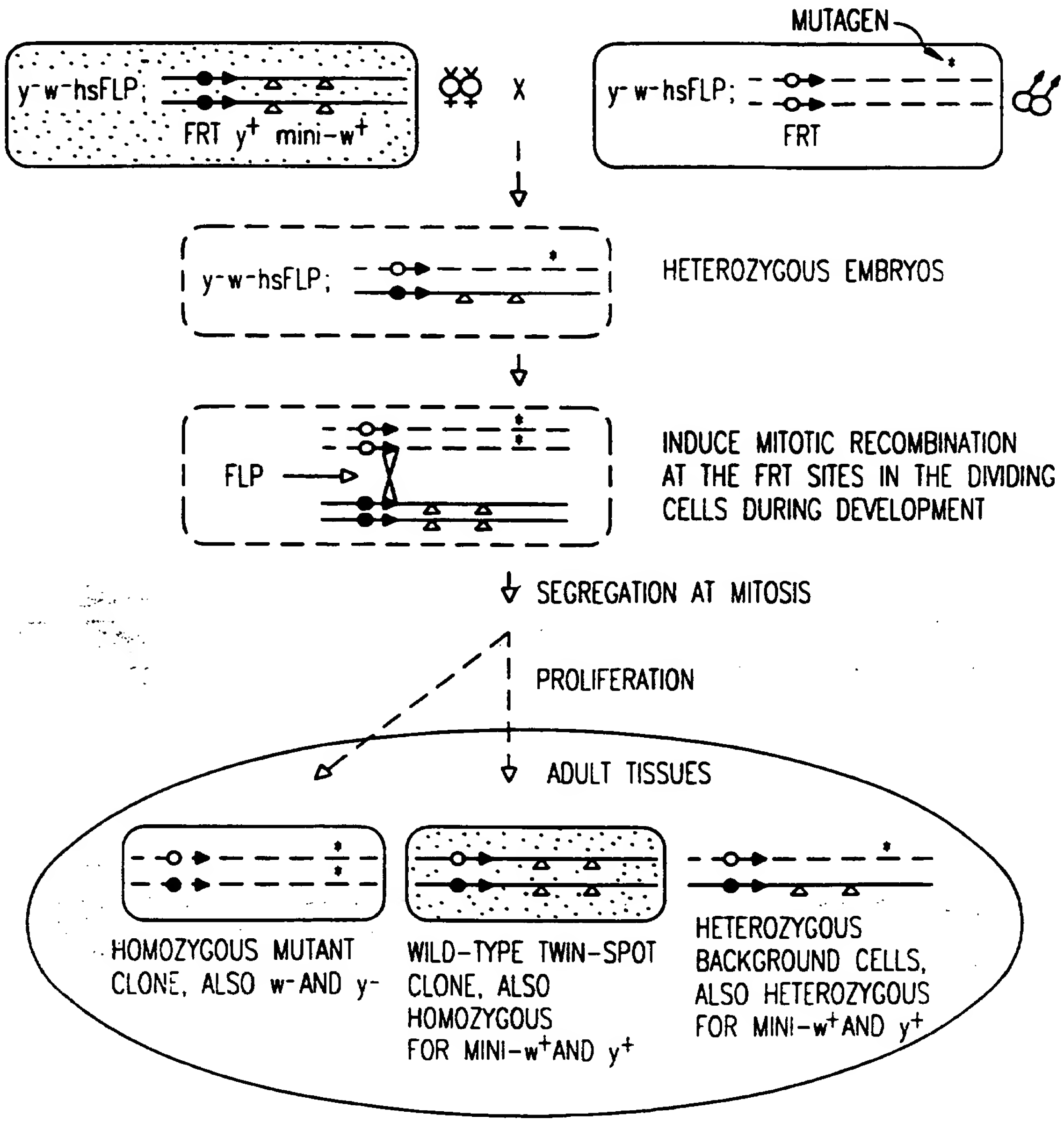
MUTAGEN
y⁻w⁻hsFLP;
FRT y+ mini-w+
X
y⁻w⁻hsFLP;
FRT
y⁻w⁻hsFLP;
HETEROZYGOUS EMBRYOS
FLP
INDUCE MITOTIC RECOMBINATION AT THE FRT SITES IN THE DIVIDING CELLS DURING DEVELOPMENT
SEGREGATION AT MITOSIS
PROLIFERATION
ADULT TISSUES
HOMOZYGOUS MUTANT CLONE, ALSO w- AND y-
WILD-TYPE TWIN-SPOT CLONE, ALSO HOMOZYGOUS FOR MINI-w+AND y+
HETEROZYGOUS BACKGROUND CELLS, ALSO HETEROZYGOUS FOR MINI-w+AND y+

FIG.1B

FIG.2C

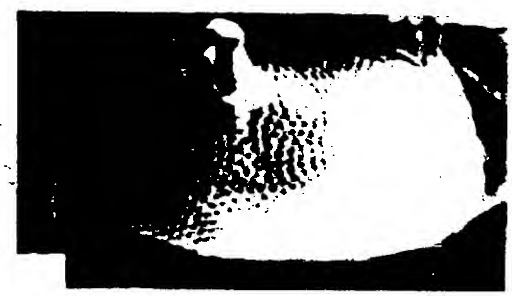

FIG.2B

FIG.2A

FIG.2F

FIG.2E

FIG.2D

FIG.2I

FIG.2H

FIG.2G

FIG.2L

FIG.2K

FIG.2J

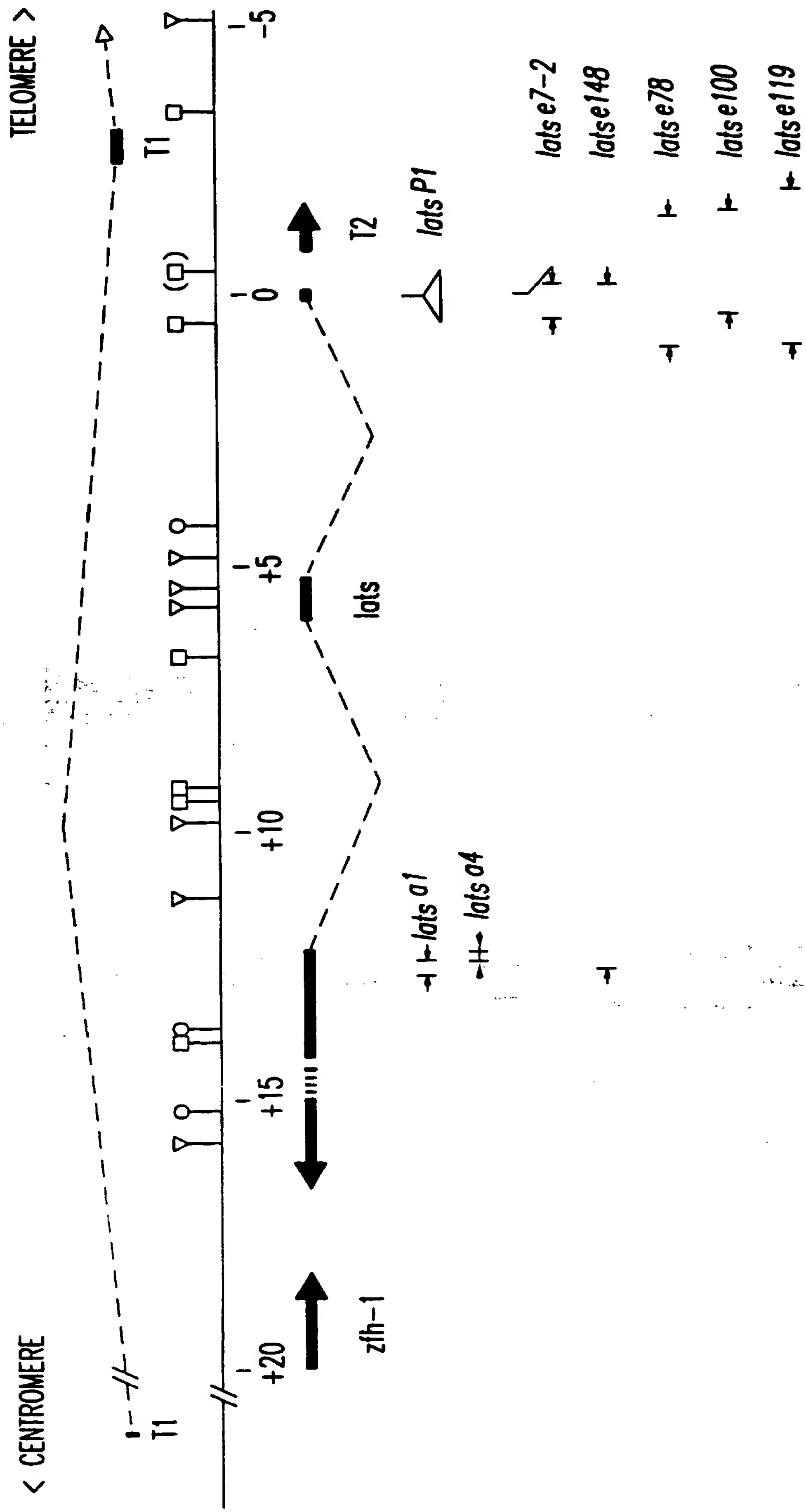
TELOMERE
CENTROMERE
T1
T2
-5
0
+5
+10
+15
+20
latsP1
lats
zfh-1
latse7-2
latse148
latse78
latse100
latse119
latsa1
latsa4

FIG.3

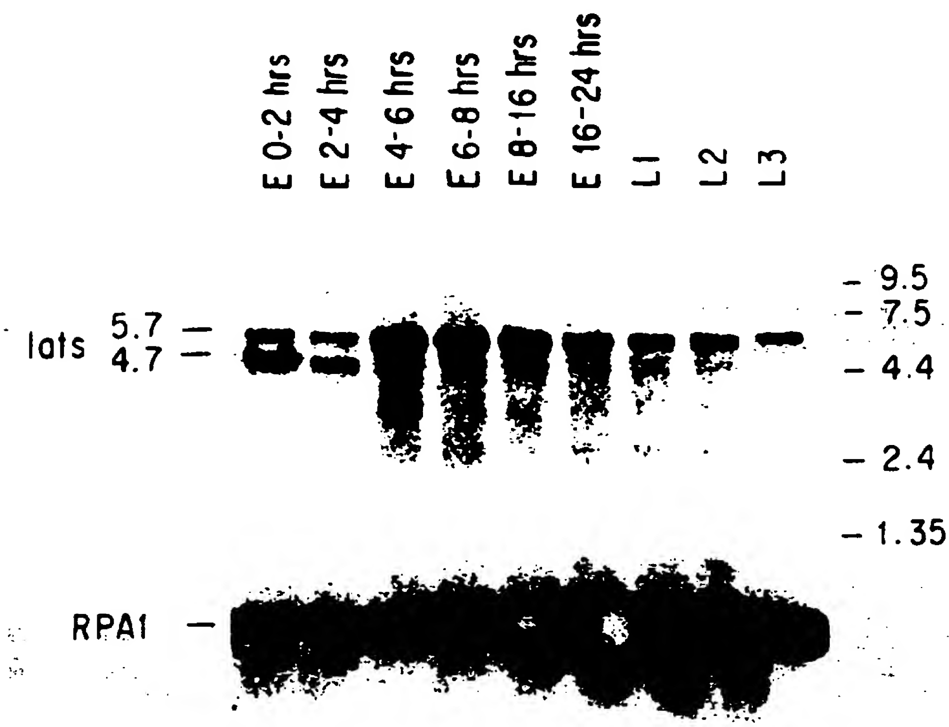
E 0-2 hrs
E 2-4 hrs
E 4-6 hrs
E 6-8 hrs
E 8-16 hrs
E 16-24 hrs
L1
L2
L3
lats
5.7
4.7
9.5
7.5
4.4
2.4
1.35
RPA1

FIG.4

```
GENOMIC
cDNA     1  ATCTAGCACGACGGCAGCAACAAAACCACGAATTAATTTTACTAAATTTAAGCCAAACGCGCATCGGAAATGCCT
                 A
        76  GAAAATGCGATTGAATGCACGCGAAAAGTGATGGGTTGCGAACGCGAGTGAATCAAGTGAAAATACGTCGGCAAA
                                                           ┌─►A2 START
       151  TATCAGCGAATTGTCGTCAAAAGGCAAGGAAAAACGGAGAAAAAGAGGAAAAGCAATAAGTGCCGTGTGTGGGAA

       226  ACGCGAAAAAGGCGAGAACAAAGAGGCGAAAAGCGAGGAAATTGCGTGGAAAACGTGGAAAACGCGAAGAAGCGA
            INTRON 1                     T
       301  AGCTCCAAGTTGGCCGCCATCGATTCGTGCGTAGGATCAATTAAGATTCCGAGTGGTCGAGAATCGGCTCAAATC

       376  AAATTAAAATCAACTAATATTTTGGTATTCAGATATTCAAATGGAATTCATTCATCGCCTGCGACTTTTATTCGG

       451  ATCTGCCAACTATTTTTGAATTTGAATTGTGTGTCTGCGGCTGGCGCAGAATCTCTGATAAAGCAGAGGAATAAA

       526  ATCGGAAGAACAACAAATACAAATACAAATGAAATGCGGGGAGCAGTATTTACATGCCAAATGAATGCTGGATAG
                GGA
       601  GCGAAAGGGGGGTTTCTCTTATAATGCAAATGTGAATGTGAATGCGAATGCGAATGCGAGTGGAAGAATTCCCG

       676  GCGCGAGTGATAAATAATCCGACGACAAACAAAGCAGAAGCCTACACCGCGAGAAAGAGCAGCGCAAACACAATT
                                                                                  G
       751  ATCTTTATTGAGAGCAACAATATCAAGATCGAGATAATAAAGCATCCTAAAACCCGCGCCTTAGTTCGTTTTAGT
                     A
       826  CTCGCCACGGATATAGATATTCAAAGGCAAAAAGGTGGTGTCGGCATCGCCAGACAAACAAGTAAAGCATCTATT

       901  TCATACAAAACAACCAATTAAATAATAATAAAAATAATAATAATCGTAGAGAGGCAGAGCCAAATCAAATTCCCG

       976  GCCGCCGATGTGCCCCAGTGTGTGTGCGTGTGTGTGTGTGTGCTGTGCTGTGCTGTGCGAGTGTTAGTGTGCG

      1051  GAGCATTTCTGTGATATGAGTGCTAAATGCCACAGGGCGAAGCAGCAGCATCATGCATCCAGCGGGCGAAAAAAG
A.A.     1                                                          M  H  P  A  G  E  K  R
      1126  GGGCGGTCGCCCCAATGATAAATACACGGCGGAAGCCCTCGAGAGCATCAAGCAGGACCTAACCCGATTTGAAGT
         8   G  G  R  P  N  D  K  Y  T  A  E  A  L  E  S  I  K  Q  D  L  T  R  F  E  V
                                 INTRON 2

      1201  ACAAAATAACCATAGGAATAATCAGAATTACACACCTCTGCGATACACGGCGACCAACGGACGCAACGATGCACT
        34    Q  N  N  H  R  N  N  Q  N  Y  T  P  L  R  Y  T  A  T  N  G  R  N  D  A  L
      1276  TACTCCTGACTATCACCACGCCAAGCAGCCGATGGAGCCGCCACCCTCCGCCTCTCCTGCTCCGGACGTGGTCAT
        59    T  P  D  Y  H  H  A  K  Q  P  M  E  P  P  P  S  A  S  P  A  P  D  V  V
                                 ──►LATS-AL DELETION
      1351  ACCGCCGCCGCCCGCCATTGTAGGTCAGCCCGGAGCCGGCTCCATATCCGTATCCGGTGTGGGCGTTGGAGTGGT
        84    P  P  P  P  A  I  V  G  Q  P  G  A  G  S  I  S  V  S  G  V  G  V  G  V  V
                                 C
      1426  GGGTGTGGCGAACGGACGTGTGCCAAAGATGATGACGGCCCTAATGCCAAACAAACTGATCCGGAAGCCGAGCAT
       109    G  V  A  N  G  R  V  P  K  M  M  T  A  L  M  P  N  K  L  I  R  K  P  S
      1501  CGAACGGGACACGGCGAGCAGTCACTACCTGCGCTGCAGTCCGGCTCTGGACTCCGGAGCCGGTAGCTCCCGATC
       134    E  R  D  T  A  S  S  H  Y  L  R  C  S  P  A  L  D  S  G  A  G  S  S  R  S
      1576  GGACAGCCCCCATTCGCACCACACCCACCAGCCGAGCTCGAGGACGGTGGGTAATCCAGGTGGAAATGGTGGATT
       159    D  S  P  H  S  H  H  T  H  Q  P  S  S  R  T  V  G  N  P  G  G  N  G  G  F
```

FIG.5A

SUBSTITUTE SHEET (RULE 26) BAD ORIGINAL

```
                                                                           G
1651 TTCTCCGTCGCCAAGCGGTTTCAGTGAGGTGGCTCCACCGGCGCCGCCGCCACGCAATCCCACCGCCTCCAGCGC
184   S  P  S  P  S  G  F  S  E  V  A  P  P  A  P  P  P  R  N  P  T  A  S  S  A
1726 GGCCACGCCCCCACCGCCAGTGCCGCCCACCAGCCAGGCGTACGTGAAGCGGCGATCACCGGCCCTGAACAACCG
209   A  T  P  P  P  P  V  P  P  T  S  Q  A  Y  V  K  R  R  S  P  A  L  N  N  R
  lot-ol deletion
1801 CCCGCCGGCGATAGCGCCACCCCACTCAGCGAGGCAACTCACCTGTSATAACCCAAAACGGGCTGAAGAACCCGC
234   P  P  A  I  A  P  P  T  Q  R  G  N  S  P  V  I  T  Q  N  G  L  K  N  P  Q
                                                                  T
1876 GCAGCAGTTGACGCAGCAGCTGAAGTCCCTGAACCTATACCCAGGCGGAGGCAGTGGAGCAGTGGTGGAGCCACC
259   Q  Q  L  T  Q  Q  L  K  S  L  N  L  Y  P  G  G  G  S  G  A  V  V  E  P  P
1951 GCCGCCCTACCTAATTCAAGGCGGAGCCGGAGGAGCAGCACCGCCGCCGCCACCACCCAGTTACACGGCCTCCAT
284   P  P  Y  L  I  Q  G  G  A  G  G  A  A  P  P  P  P  P  P  S  Y  T  A  S  M
2026 GCAGTCGCGGCAGTCGCCCACACAATCCCAACAATCGGACTACAGGAAATCCCCGAGCAGTGGGATATACTCGGC
309   Q  S  R  Q  S  P  T  Q  S  Q  Q  S  D  Y  R  K  S  P  S  S  G  I  Y  S  A
                                          C
2101 CACCTCGGCGGGCTCGCCGAGCCCCATAACTGTGTCGCTGCCGCCGGCGCCGCTGGCGAAGCCACAACCACGAGT
334   T  S  A  G  S  P  S  P  I  T  V  S  L  P  P  A  P  L  A  K  P  Q  P  R  V
2176 CTACCAGGCCAGGAGTCAGCAGCCGATCATCATGCAGAGTGTGAAGAGCACGCAGGTCCAAAAGCCCGTGCTGCA
359   Y  Q  A  R  S  Q  Q  P  I  I  M  Q  S  V  K  S  T  Q  V  Q  K  P  V  L  Q
2251 AACAGCAGTGGCGCCCCAATCGCCATCGAGTGCCTCGGCCAGCAATTCACCAGTCCACGTGCTGGCCGCTCCACC
384   T  A  V  A  P  Q  S  P  S  S  A  S  A  S  N  S  P  V  H  V  L  A  A  P  P
2306 CTCTTACCCTCAGAAGTCCGCGGCAGTGGTGCAGCAGCAGCAACAGGCAGCAGCGGCGGCCCACCAGCAGCAGCA
409   S  Y  P  Q  K  S  A  A  V  V  Q  Q  Q  Q  Q  A  A  A  A  A  H  Q  Q  Q  H
                                     C                   T
2401 TCAGCACCAGCAATCCAAACCACCAACGCCAACCACACCGCCCTTGGTGGGTCTGAACAGCAAGCCCAATTGCCT
434   Q  H  Q  Q  S  K  P  P  T  P  T  T  P  P  L  V  G  L  N  S  K  P  N  C  L
2476 GGAGCCACCGTCCTATGCCAAGAGCATGCAGGCCAAGGCGGCCACGGTGGTACAGCAGCAGCAACAGCAGCAGCA
459   E  P  P  S  Y  A  K  S  M  Q  A  K  A  A  T  V  V  Q  Q  Q  Q  Q  Q  Q  Q
   AAC
 G  G  A                                G
2551 ACAACAGCAGGTCCAGCAGCAGCAGGTGCAACAGCAGCAGCAACAGCAGCAACAGCAACTGCAGGCCTTGAGGGT
484   Q  Q  Q  V  Q  Q  Q  Q  V  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  L  Q  A  L  R  V

          GGGAGCGGGATCAAC
2626 GCTCCAGGCACAGGCTCAGAGGGAGCGGGATCAACGGGAGCGGGAACGGGATCAGCAGAAGCTGGCCAACGGAAA
559   L  Q  A  Q  A  Q  R  E  R  D  Q  R  E  R  E  R  D  Q  Q  K  L  A  N  G  N

2701 TCCTGGCCGGCAGATGCTTCCGCCGCCGCCCTATCAGAGCAACAACAACAACAACAGCGAGATCAAACCGCCGAG
534   P  G  R  Q  M  L  P  P  P  P  Y  Q  S  N  N  N  N  N  S  E  I  K  P  P  S

2776 CTGCAACAACAACAACATACAGATAAGCAACAGCAACCTGGCGACGACACCACCCATTCCGCCTGCCAAATACAA
559   C  N  N  N  N  I  Q  I  S  N  S  N  L  A  T  T  P  P  I  P  P  A  K  Y  N

2851 TAACAACTCCTCCAACACGGGCGCGAATAGCTCGGGCGGCAGCAACGGATCCACCGGCACCACCGCCTCCTCGTC
584   N  N  S  S  N  T  G  A  N  S  S  G  G  S  N  G  S  T  G  T  T  A  S  S  S

2926 GACCAGCTGCAAGAAGATCAAGCACGCCTCGCCCATCCCGGAGCGCAAGAAGATCTCCAAGGAGAAGGAGGAGGA
609   T  S  C  K  K  I  K  H  A  S  P  I  P  E  R  K  K  I  S  K  E  K  E  E  E
```

FIG.5B

3001 GCGCAAGGAGTTCCGCATCAGGCAGTACTCGCCGCAAGCCTTCAAGTTCTTCATGGAGCAGCACATAGAGAACGT
634 R K E F R I R Q Y S P Q A F K F F M E Q H I E N V

3076 GATCAAGTCGTATCGCCAGCGCACGTATCGCAAGAATCAGCTGGAGAAGGAGATGCACAAAGTGGGACTGCCCGA
709 I K S Y R Q R T Y R K N Q L E K E M H K V G L P D

3151 TCAGACCCAAATCGAGATGAGGAAAATGCTGAACCAAAAGGAGAGCAACTACATTCGATTGAAGCGCGCCAAGAT
684 Q T Q I E M R K M L N Q K E S N Y I R L K R A K M

3226 GGACAAGAGCATGTTCGTCAAACTGAAGCCCAATTGGAGTGGGTGCATTTGGCGAGGTAACGCTGGTGAGCAAAT
759 D K S M F V K L K P I G V G A F G E V T L V S K I
3301 CGATACCTCGAACCATTTGTATGCGATGAAAACCCTGCGGAAAGCGGACGTTCTCAAGCGGAATCAGGTGGCACA
734 D T S N H L Y A M K T L R K A D V L K R N Q V A H

3376 CGTGAAGGCCGAGAGGGATATCCTCGCGGAAGCCGACAATAACTGGGTGGTGAAGTTGTACTACAGCTTCCAGGA
809 V K A E R D I L A E A D N N W V V K L Y Y S F Q D

Intron 3

3451 CAAGGATAATCTGTACTTTGTGATGGACTACATACCAGGTGGTGATCTGATGTCTCTGCTCATCAAACTGGGCAT
784 K D N L Y F V M D Y I P G G D L M S L L I K L G I

3526 TTTCGAGGAGGAACTGGCCAGATTCTACATCGCCGAGGTCACCTGCGCCGTGGACAGCGTTCACAAAATFFFCTT
809F F E E E L A R F Y I A E V T C A V D S V H K M G F

Intron 4

3601 CATTCACAGAGACATCAAGCCTGACAACATACTCATCGATAGGGACGGACACATAAAGCTCACCGACTTTGGCCT
834 I H R D I K P D N I L I D R D G H I K L T D F G L

Intron 5

3676 GTGCACGGGATTCCGATGGACGCACAACTCGAAGTACTACCAGGAGAACGGCAATCACTCGCGCCAGGACTCGAT
859 C T G F R W T H N S K Y Y Q E N G N H S R Q D S M

Intron 6

3751 GGAGCCCTGGGAGGAATACTCCGAGAACGGACCGAAGCCCACCGTGCTGGAGAGGCGACGGATGCGCGATCACCA
884 E P W E E Y S E N G P K P T V L E R R R M R D H Q

A

3826 AAGAGTCCTGGCCCACTCGCTGGTGGGCACCCCGAACTACATAGCTCCCGAGGTGCTGGAGAGGAGTGGGTACAC
909 R V L A H S L V G T P N Y I A P E V L E R S G Y T

C T

3901 GCAGCTGTGCGACTACTGGAGCGTGGGCGTCATCCTTTACGAGATGCTGGTGGGTCAGCCGCCCTTTCTGGCCAA
934 G L C D Y W S V G V I L Y E M L V G Q P P F L A N

Intron 7 C

3976 CAGTCCGCTGGAAACGCAACAAAAGGTCATCAACTGGGAGAAAACGCTGCATATTCCGCCGCAGGCCGAGTTATC
959 S P L E T Q Q K V I N W E K T L H I P P Q A E L S

4051 CCGCGAGGCTACGGACTTGATAAGGAGGCTCTGTGCGTCGGCTGACAAGCGGCTGGGCAAGAGCGTGGACGAGGT
984 R E A T D L I R R L C A S A D K R L G K S V D E V

4126 CAAGAGCCACGACTTCTTCAAGGGCATCGACTTTGCGGACATGCGGAAGCAGAAAGCGCCCTACATACCGGAAAT
1059 K S H D F F K G I D F A D M R K Q K A P Y I P E I

FIG.5C

BAD ORIGINAL

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                G
4201  CAAGCACCCAACGGACACATCCAACTTTGATCCCGTGGATCCGGAGAAGCTGCGCTCGAATGACTCCACCATGAG
1034   K  H  P  T  D  T  S  N  F  D  P  V  D  P  E  K  L  R  S  N  D  S  T  M  S
                                     C     C
4276  CAGCGGCGATGATGTCGATCAGAATGACCFCACTTTCCACGGCTTTTTCGAATTTACCTTCCGTCGCTTCTTCGA
1059   S  G  D  D  V  D  Q  N  D  R  T  F  H  G  F  F  E  F  T  F  R  R  F  F  D

4351  CGACAAGCAGCCGCCGGATATGACGGACGATCAGGCGCCGGTTTACGTCTGAAATGGATGCTCTCCATGTGCCCA
1084   D  K  Q  P  P  D  M  T  D  D  Q  A  P  V  Y  V

4426  ACACCAACACCCCGCCCCCGAATCATTGTTAGTCAAATAGTCACAAAAAGGGGATAGAAACCATTGAGTGGGCTT

4501  GCATTGTAAAGGAAGCCTGGGTATAGAATGAAACTATCTATATACATTATATAAATTATAGGAGACAGTAGAGGC

4576  GGAGCTACGTATATACATACAAATAATATACATATATTTGATATATATATATATATATATGCCGTAGGGCATGA

4651  ACTGAATAAATATAAAACGGAGCCGAGTAGAGATGAAACGAGAGGAGCGAGTCAGGACCTTCGACCTTTAACTGA
         Poly A                                                               G
4726  ACATAGTATATCCTTGTGCACTACTACTCCACAACAAATATATATTTTTAAATTGTTAGAATTCAAAAGGGACCA
                                 delete                                      C
4801  ACTGGAAATCGAACCTTTCTGGTGCTCAAAGCAAAGCAAAGCAAAGCAAAACAAAACGCCTTAAACTAAATGAGA

4876  CGCGAATTTACCCAACCACTTCACTCCTCTCCTTTCTCCACCTCCGATCGGTGGCCGGATTCGAACTCAGCAGGC
                              T
4951  TGGTTGCATCCGGCCATCCCATTGACTTCCCATTCAGAATTGAGATTGCGAGGTGTGCGATGGAGAACGAACGGA

5026  GACCAAAAGTCGCACGGCAGCGATATAAGCGGGTCTTATAAGCCCTAATCTAAATCTAAACTGGGAGAACAGGACC

                                                   GTGGCCCCCCTCCCTCCCTCCTCAT
      C  TGTAATTAGTG                         A   A
5101  TATGTATGTCCTGCTATCCAATTCGTCTATCACTGCTCTTCATCTGTGTACGACCCCCACCCCCCCCCTCCCCAT

            Identical to the 1-141 n.t. of the Drosophila plc-21 transcript
5176  CCAAAAGAACAAACTTAGACGTAGCCTATGTGAAAAGCTAGCAATGTTAGACCAACTTGTTGAATGCCAAATGAA
                                                                             de
5251  ATTGTTTAGCCCCATGAGGAAAACGCGGGGGAAATTCAACACTTATTCTCTGATAGCAAACGGAAAAGAAAGAAA
      ete
5326  GAAAAAAAAAAACAGAAACAGTACGAGAAAATTGTAATCTTCTTAATGTAATATTGTAAAFAACACFRRAARRGT

5401  AATCTATGCTAGAGTTGTGTAGCGCCCTAAGATGTTTTTTAGTTTATAGACCGCTAACCGTAATCTAGTTTAATT

5476  CCTAACACTAAGCGAGAGTACAGTACATTGGTTTTTTTGTTTGTCGTAGGTTCGTTGGAAAATGCTTAACGGGAA

5551  ACGATTTGTTTTTCTCTTTAATTAGCTTCAGTTTGTATGTGCGTGTGTTTTTATTATGACTTATATATAGTCCAT

5626  CTGAATATTCGTGGATGGAGCCTATTTTAAATGTGAGATCGAGCTAATTGAAGGAAATACAAACAAACTCTGTGT
                           AAAAGCAAATTAATAAAT
5701  GCCTAGGCCAATTAGTTAT                    
                                Poly A
```

BAD ORIGINAL

FIG.5D

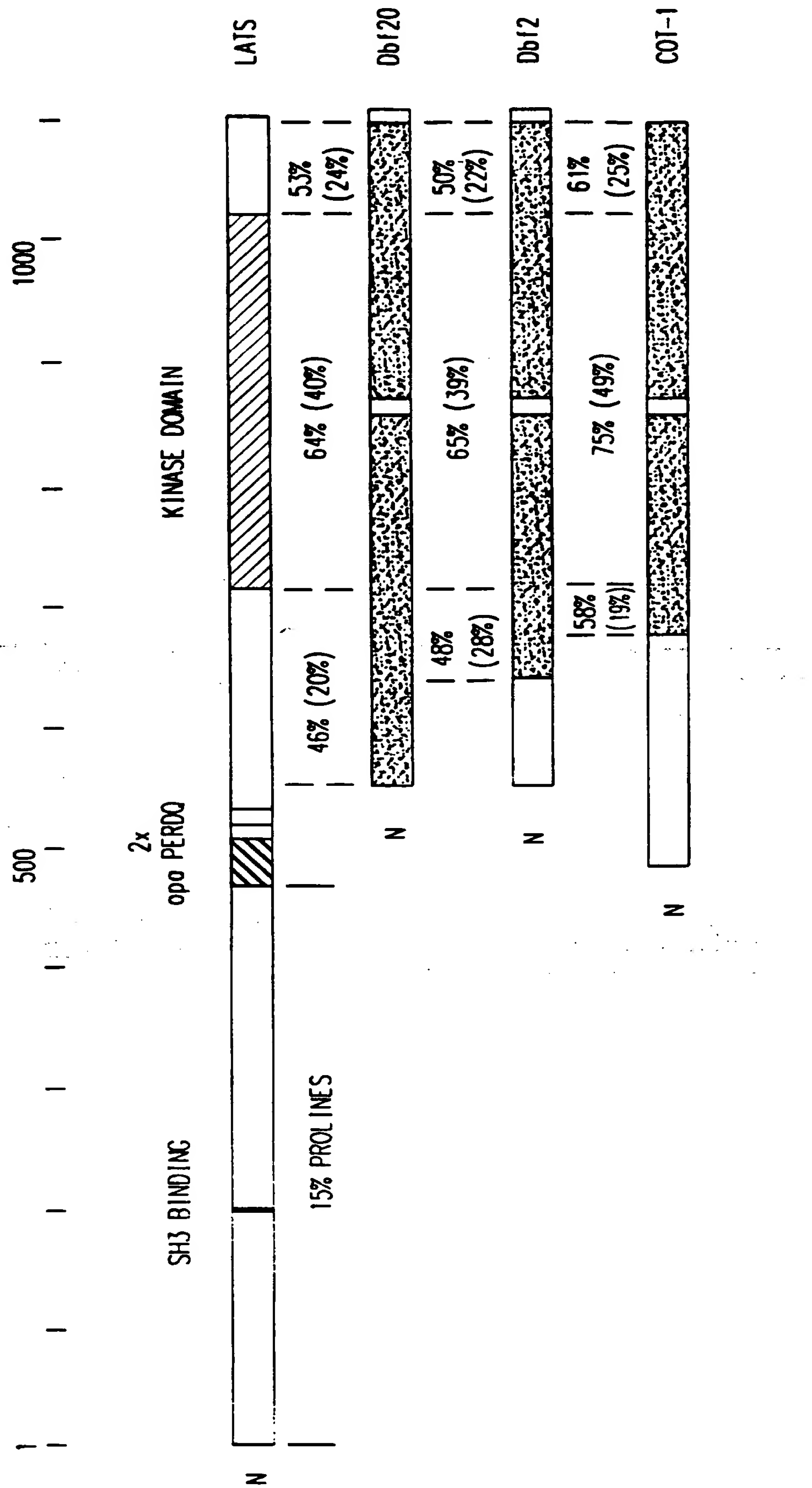

LATS
Dbf20
Dbf2
COT-1
1000
500
1
KINASE DOMAIN
2x
opa PERDQ
SH3 BINDING
15% PROLINES
53%
(24%)
64% (40%)
46% (20%)
50%
(22%)
65% (39%)
48%
(28%)
61%
(25%)
75% (49%)
58%
(19%)
N
N
N
N

FIG.6A

```
LATS  DROSOPHILA 546 SNNNNNSEIKPPSQNNNNIQISNSNLATTPPIPPAKYN-NNSSNTGANSSGGSNGSTGTTASSSTSQKKI--KHASPIPERKKISKEKEEERKEFRIRQYS
PKTL7 TOBACCO     |                                                                      MDSARGWFQKLSSTKKD-----PMASGREDQKPVSAEEASNITKQRV
PK    SPINACH     |                                                                                                                 MEK
DBF20 YEAST       |  MFSRSDREVDDLAGNMSHLGFYDLNIR-KPTSPQAQYRPARKSENGRLTPGLPRSYKPCDSDDQDTFKNRISLNHSPKKLPK-DFHERASQSKTQRVVNVC
DBF2  YEAST      82                                                                                                  ERATSNKTQRVVSVC

                                                                                  KINASE DOMAIN
LATS DROSOPHILA  644 PQAFI-KTFMEQHIENVIKSYRQ-RTYRKNQ-LEKEMHKVGLPDQTQIEM---RKMLNQKESNYIRLKRAKMDKSMFVKLKPIGVGAFGEVTLVS-KIDTS
COT-1 NEUROSPORA 191                    FRQSE-MEQKLGETN-DARRRESI---WSTAGRKEGQYLRFLRTKDKPENYQTIKIIGKGAFGEVKLVQ-K-KAD
PKTL7  TOBACCO    43 ANA  KQYIEKHYREQMKN-LQERRERRIL-LEKKLADADVSEEDQNNL---LKFLEKKETEYMRLQRHKMGADDFELLTMIGKGAFGEPICMIGFSVITG
PK COMMON
ICE PLANT          1                           RKLHDADVSEEDQNNL---LKFLEKKETKYMRLQRHKMGADDFELLTMIGKGAFGEVR-VC-REKTT
PK SPINACH         4 VKAA-KKFIENHYRSQMKN-IQERKERRWV-LEKQLASSDVPEEEQMSL---IKDLERKETEFMRLKRNRICVNDFELLTIIGRGAYGEVQL-C-REKKS
DBF20   YEAST    100 QLYFLDYYCDM-FDYVI-SRRQ-RTKQVLRYLEQQRSVKNVSNKVLNEE---WALYLQREHEVLRKRRLKPKHKDFQILTQVGQGGYGQVYLAK-KKD-S
DBF2    YEAST     97 KMYFLEYYCDM-FDYVI-SRRQ-RT--KQV-LEYLQQQSQLPNSDQIKLNEEWSSYLQREHQVLRKRRLKPKNRDFEMITQVGQGGYGQVYLAR-KKDT-

LATS DROSOPHILA  737 NH          LYAMKTLRKADVLKRNQVAHVKAERDILAEADNNWVVKLYYSFQDKDNLYFVMDYIPGGDLMSLLIKLGTFEFELARFYIAEVTCAVDSVH
COT-1 NEUROSPORA 259 GK          VYAMKSLIKTEMFKKDQLAHVRAERDILAESDSPWVVKLYTTFQDANFLYMLMEFLPGGDLMTMLIKYETESEDITRFYIAEIVLAIDAVH
PKTL7 TOBACCO    137 QNCREKTTGQVYAMKKLKKSEMLRRGQVEHVKAERNLLAEVDSDCIVKLYYSFQDDDYLYLVMEYLPGGDMMTLLMRKDTLTEDEARFYVAETVLAIESIH
PK   COMMON
ICE  PLANT        63 GH      ----VYAMKKLKKSEMLRRGQVEHVKAERNLLAEVDSNCIVKLYCSFQDEEYLYLIMEYLPGGDMMTLLMRKDTLTEDEARFYVAETILAIESIH
PK   SPINACH      96 EN        --IYAMKKLKKSEMLSRGQVEHVRAERNLLAEVDSHCIVKLFYSFQDAEYLYLIMEYLPGGDMMTLLMREDTLTEKVAKFYIAQSVLAIESIH
DBF20     YEAST  193 DE          ICALKILNKKLLFKLNETNHVLTERDILTTTRSDWLVKLLYAFQDPESLYLAMEFVPGGDFRTLLINTRILKSGHARFYISEMFCAVNALH
DBF2      YEAST  190 KE        --VCALKILNKKLLFKLNETKHVLTERDILTTTRSEWLVKLLYAFQELQSLYLAMEFVPGGDFRTLLINTRCLKSGHARFYISEMFCAVNALH
```

FIG.6B

```
LAIS DROSOPHILA   830 KMGFIIRDIKPDNILIDRDGHIKLTDFGLCTGFRW-THNSKYYQENG-NHSRQDSMEPWEEYSENGPKPT-VLERRRM---RDHQRMLAHSLVGTPNYIAP
COT-1 NEUROSPORA  352 KLGFIIRDIKPDNILLDRGGHVKLTDFGLSTGFHK-LHDNNYYTQLL-QGKSNKPRDNRNSVAIDQINLT-VSNRAWINDWRRSRRLMAYSTVGTPDYIAP
PKIL7 TOBACCO     238 KIINYIHRDIKPKNLLLDRYGHLKLSDFGLCKPLDCSTLEEKDF-SVG-DNANGGSRSDSPP-APKRTQQE-QLEHWQ--KNR---RMLAYSTVGTPDYIAP
PK COMMON
ICE PLANT         156 KIINYIHRDIKPDNLLLDKFGHLRLSDFGLCKPLDCSTLEEKDFEVNN-GNGGSPSNEGSTK-PRR-TQQE-QLQHWQ--KNR---RMLAYSTVGTPDYIAP
PK SPINACH        189 KIINYIHRDIKPDNLLLDKNGHMLLSDFGLCKPLDC-ATLSTIKENESMDDVSKNSMDIDASLPDAGNGHSWRSAREQLQHWQRNRRKLAFSTVGTPDYIAP
DBF20 YEAST       285 ELGYIHRDLKPENFLIDATGHIKLTDFGLAAGT---VSNERIESMKI-RLEEVKNLQ-FPAFTERSIEDR-SKIYHNM---RKTEINYANSMVGSPDYMAL
DBF2 YEAST        282 DLGYIHRDLKPENFLIDAKGHIKLTDFGLAAGT---ISNERIESMKI-RLEKIKDLE-FPAFTEKSIEDR-RKMYNQL---REKEINYANSMVGSPDYMAL

                                                                                          KINASE DOMAIN

LAIS DROSOPHILA   925 EVIERSGYTQLCDYWSVGVILYEMLVGQPPFLANSPLETQQKVINWEKTLHIP---PQAELSREATDLI-RRLCASADKRLG-KSVDEVKSHDFFKGIDE
COT-1 NEUROSPORA  450 EIFIGIGYSFDCDWWSLGTIMFECLVGWPPFCAEDSHDTYRKIVNWRHSLYFP---DDITLGVDAWNLI-RSLICNTENRLGRGGAHEIKSHAFFRGVEF
PKIL7 TOBACCO     330 EVIIKKGYGMECDWWSLGAIMYEMLVGYPPFYSDDPMSTCRKIVNWKNHLKFP---EEAKLSPEAKDLISRLLCNVTE-RLGSNGADEIKVHSWFKGTDW
PK COMMON
ICE PLANT         248 EVILKKGYGMECDWWSLGAIMYEMLVGYPPFYSDDPMSTCRKIVNWRTHLKFP---EEAKLSPEAKDLISKLLCNVTQ-RLGSNGAHEIKLHPWFNGIDW
PK SPINACH        289 EVILKKGYGMECDWWSLGAIMYEMLVGYPPFYSDDPITTCRKIVHWRHYLKFP---DDAKLTFEARDLICRLLC-DVEHRLGTGGAEQIKVHAWFKDVEW
DBF20 YEAST       377 EVLEGKKYDFTVDYWSLGCMLFESLVGYTPFSGSSTNETYENLRYWKKTLRRPRTEDRRAAFSDRTWDLI-TRLIADPINRV--RSFEQVRKMSYFAEINF
DBF2 YEAST        374 EVLEGKKYDFTVDYWSLGCMLFESLVGYTPFSGSSTNETYDNLRRWKQTLRRPRQSDGRAAFSDRTWDLI-TRLIADPINRL--RSFEHVKRMSYFADINF

LAIS DROSOPHILA  1020 ADMRKQKAPYIPEIKHPTDTSNFDPVDPEKLRSNDSTMSSGDDVDQ--------ND-RTFHGFFEFTFRRFF--DDKQPPDMIDDQAPVYV.
COT1-NEUROSPORA   546 DSLRRIRAPFEPRLTSAIDTTYF-PTD-EIDQTDNATLLKAQQAARGAAAPAQQEESPELSPFIGYTFKRF---DNNFR.
PKIL7 TOBACCO     426 DRIYQMEAAFIPEVNDELDTQNFEKFE-ESESHSQSGSRSGPWRKML---------SSKDINFVGYTYKNFKVVNDYQVPGMVELKKTNTK + 20 A.A.
PK COMMON
ICE PLANT         344 LRIYQMEAAFIPEVNDELDTQNFEKFE-EADNSSQSTSKAGPWRKML---------SSKDLNFVGYTYKNFEIVNDYQVPGIAELKKKQTK + 55 A.A.
PK SPINACH        385 DRIYEIDAAYKPQVNGELDTQNFMKFD-EANPPTPSRSGSGPSRKML---------TSKDLSFVGYTYKNF---DAVKGLKHSFDRKGSTS + 39 A.A.
DBF20 YEAST       475 EIIRISSPPFIPQLDDETDAGYFDDFTNEEDMAKYADVFKRQNKLSAMV-----DDSAVDSKLVGFTFRHR---DGKQGSSGILYNGSEHSDPFSTFYD.
DBF2 YEAST        472 SIIRSMIPPFIPQLDSETDAGYFDDFTSEADMAKYADVFKRQDKLTAMV-----DDSAVSSKLVGFTFRHR---NGKQGSSGILFNGLEHSDPFSTFYD.
```

FIG.6C

```
        10         20         30         40         50         60         70         80
         *          *          *          *          *          *          *          *
GTGCAACATT CAATTAACCG AAAACAAAGC TGGAAAGGTT CTAAAGAGTC TCTAGTTCCT CAGAGACACG GCCCATCTCT
 V  Q  H   S  I  N  R   K  Q  S   W  K  G   S  K  E  S   L  V  P   Q  R  H   G  P  S  L
        90        100        110        120        130        140        150        160
         *          *          *          *          *          *          *          *
AGGAGAAAAT GTGGTTTATC GTTCTGAAAG CCCCAACTCA CAGGCGGATG TAGGAAGACC TCTGTCTGGA TCCGGCATTG
 G  I  N   V  V  Y   R  S  E  S   P  N  S   Q  A  D   V  G  R  P   L  S  G   S  G  I
       170        180        190        200        210        220        230        240
         *          *          *          *          *          *          *          *
CAGCATTGCC TCAAGCTCAC CCAAGCAATG GACAGAGAGT GAACCCCCCA CCACCACCTC AAGTTAGGAG TGTTACTCCT
A  A  I  A   Q  A  H   P  S  N   G  Q  R  V   N  P  P   P  P  P   Q  V  R  S   V  T  P
       250        260        270        280        290        300        310        320
         *          *          *          *          *          *          *          *
CCACCACCTC CGAGAGGCCA GACCCCACCT CCCCGAGGCA CCACTCCCCC TCCCCCCTCA TGGGAACCAA GCTCTCAGAC
 P  P  P   P  R  G  Q   T  P  P   P  R  G   T  T  P  P   P  P  S   W  E  P   S  S  Q  T
       330        340        350        360        370        380        390        400
         *          *          *          *          *          *          *          *
AAAGCGCTAC TCTGGGAACA TGGAGTACGT AATCTCCCGA ATCTCCCCTG TTCCACCTGG GGCGTGGCAG GAGGGGTACC
 K  R  Y   S  G  N   M  E  Y  V   I  S  R   I  S  P   V  P  P  G   A  W  Q   E  G  Y
       410        420        430        440        450        460        470        480
         *          *          *          *          *          *          *          *
CTCCACCACC TCTTACCACT TCTCCCATGA ATCCCCCTAG CCAGGCTCAG AGGGCCATTA GTTCTGTTCC AGTTGGTAGA
P  P  P  P   L  T  T   S  P  M   N  P  P  S   Q  A  Q   R  A  I   S  S  V  P   V  G  R
       490        500        510        520        530        540        550        560
         *          *          *          *          *          *          *          *
CAACCCATCA TCATGCAGAG TACTAGCAAA TTTAACTTTA CACCAGGGCG ACCTGGAGTT CAGAATGGTG GTGGTCAGTC
 Q  P  I   I  M  Q  S   T  S  K   F  N  F   T  P  G  R   P  G  V   Q  N  G   G  G  Q  S
```

FIG. 7A

BAD ORIGINAL

```
       570        580        590        600        610        620        630        640
         *          *          *          *          *          *          *          *
TGATTTTATC GTGCACCAAA ATGTCCCCAC TGGTTCTGTG ACTCGGCAGC CACCACCTCC ATATCCTCTG ACCCCAGCTA
 D  F  I   V  H  Q   N  V  P  T   G  S  V   T  R  Q   P  P  P  P   Y  P  L   T  P  A
       650        660        670        680        690        700        710        720
         *          *          *          *          *          *          *          *
ATGGACAAAG CCCCTCTGCT TTACAAACAG GGGCTTCTGC TGCTCCACCA TCATTCGCCA ATGGAAACGT TCCTCAGTCG
N  G  Q  S   P  S  A   L  Q  T   G  A  S  A   A  P  P   S  F  A   N  G  N  V   P  Q  S
       730        740        750        760        770        780        790        800
         *          *          *          *          *          *          *          *
ATGATGGTGC CCAACAGGAA CAGTCATAAC ATGGAGCTTT ATAATATTAA TGTCCCTGGA CTGCAAACAG CCTGGCCCCA
 M  M  V   P  N  R  N   S  H  N   M  E  L   Y  N  I  N   V  P  G   L  Q  T   A  W  P  Q
       810        820        830        840        850        860        870        880
         *          *          *          *          *          *          *          *
GTCGTCTTCT GCTCCTGCGC AGTCATCCCC AAGCGGTGGG CATGAAATTC CTACATGGCA ACCTAACATA CCAGTGAGGT
 S  S  S   A  P  A   Q  S  S  P   S  G  G   H  E  I   P  T  W  Q   P  N  I   P  V  R
       890        900        910        920        930        940        950        960
         *          *          *          *          *          *          *          *
CAAATTCTTT AATAACCCCA TTAGGAAGTA GAGCAAGTCA CTCTGCTAAT TCTCAGCCTT CTGCCACTAC AGTCACTGCC
S  N  S  I   N  N  P   L  G  S   R  A  S  H   S  A  N   S  Q  P   S  A  T  T   V  T  A
       970        980        990       1000       1010       1020       1030       1040
         *          *          *          *          *          *          *          *
ATCACACCCG CTCCTATTCA ACAGCCCGTG AAAAGCATGC GCGTCCTGAA ACCAGAGCTG CAGACTGCTY TAGCCCCAAC
 I  T  P   A  P  I  Q   Q  P  V   K  S  M   R  V  L  K   P  E  L   Q  T  A   L  A  P  T
      1050       1060       1070       1080       1090       1100       1110       1120
         *          *          *          *          *          *          *          *
CCATCCTTCT TGGATGCCAC AGCCAGTTCA GACTGTTCAG CCTACCCCTT TTTCTGAGGG TACAGCTTCA AGTGTGCCTG
 H  P  S   W  M  P   Q  P  V  Q   T  V  Q   P  T  P   F  S  E  G   T  A  S   S  V  P
```

FIG. 7B

BAD ORIGINAL

```
      1130       1140       1150       1160       1170       1180       1190       1200
         *          *          *          *          *          *          *          *
TCATCCCACC TGTTGCTGAA GCTCCAAGCT ATCAAGGTCC ACCACCGCCT TATCCAAAAC ATCTGCTACA CCAAAACCCA
V  I  P  P   V  A  E   A  P  S   Y  Q  G  P   P  P  P   Y  P  K   H  L  L  H   Q  N  P
      1210       1220       1230       1240       1250       1260       1270       1280
         *          *          *          *          *          *          *          *
TCTGTCCCTC CATATGAGTC AGTAAGTAAG CCCTGCAAAG ATGAACAGCC TAGCTTACCC AAGGAAGATG ATAGTGAGAA
 S  V  P   P  Y  E  S   V  S  K   P  C  K   D  E  Q  P   S  L  P   K  E  D   D  S  E  K
      1290       1300       1310       1320       1330       1340       1350       1360
         *          *          *          *          *          *          *          *
GAGTGCGGAC AGTGGTGACT CTGGGGATAA AGAAAAGAAA CAGATTACAA CTTCACCTAT CACTGTTCGG AAAAACAAGA
 S  A  D   S  G  D   S  G  D  K   E  K  K   Q  I  T   T  S  P  I   T  V  R   K  N  K
      1370       1380       1390       1400       1410       1420       1430       1440
         *          *          *          *          *          *          *          *
AAGATGAAGA ACGAAGAGAG TCTCGGATTC AGAGTTACTC CCCACAGGCC TTTAAGTTCT TCATGGAGCA GCACGTAGAG
K  D  E  E   R  R  E   S  R  I   Q  S  Y  S   P  Q  A   F  K  F   F  M  E  Q   H  V  E
      1450       1460       1470       1480       1490       1500       1510       1520
         *          *          *          *          *          *          *          *
AACGTCCTGA AGTCTCATCA GCAGCGTCTG CATCGGAAGA AGCAGCTAGA AAATGAAATG ATGCGGGTTG GATTATCTCA
 N  V  I   K  S  H  Q   Q  R  L   H  R  K   K  Q  L  E   N  E  M   M  R  V   G  L  S  Q
      1530       1540       1550       1560       1570       1580       1590       1600
         *          *          *          *          *          *          *          *
AGATGCCCAG GATCAAATGA GAAAGATGCT TTGCCAGAAA GAGTCTAACT ATATTCGTCT TAAAAGGGCT AAAATGGACA
 D  A  Q   D  Q  M   R  K  M  L   C  Q  K   E  S  N   Y  I  R  L   K  R  A   K  M  D
      1610       1620       1630       1640       1650       1660       1670       1680
         *          *          *          *          *          *          *          *
AGTCTATGTT TGTAAAGATA AAGACATTAG GAATAGGAGC GTTTGGTGAA GTCTGTCTAG CAAGAAAAGT CGATACTAAA
K  S  M  I   V  K  I   K  T  L   G  I  G  A   F  G  E   V  C  L   A  R  K  V   D  T  K
```

FIG. 7C

BAD ORIGINAL

```
      1690      1700      1710      1720      1730      1740      1750      1760
         *         *         *         *         *         *         *         *
GCTTTGTATG CAACAAAGAC TCTTCGAAAG AAAGACGTTC TGCTCCGAAA TCAGGTGGCT CATGTGAAAG CGGAGAGGGA
 A  L  Y   A  T  K  I   L  R  K   K  D  V   L  L  R  N   Q  V  A   H  V  K   A  E  R  D
      1770      1780      1790      1800      1810      1820      1830      1840
         *         *         *         *         *         *         *         *
TATCCTAGCA GAAGCCGACA ATGAGTGGGT GGTCCGCCTG TACTACTCTT TCCAGGACAA GGACAACTTG TACTTTGTGA
  I  L  A   E  A  D   N  E  W  V   V  R  L   Y  Y  S   F  Q  D  K   D  N  L   Y  F  V
      1850      1860      1870      1880      1890      1900      1910      1920
         *         *         *         *         *         *         *         *
TGGACTACAT TCCTGGGGGG GATATGATGA GCCTATTAAT TAGAATGGGC ATCTTTCCTG AAAATCTGGC ACGATTCTAC
 M  D  Y  I   P  G  G   D  M  M   S  L  L  I   R  M  G   I  F  P   E  N  L  A   R  F  Y
      1930      1940      1950      1960      1970      1980      1990      2000
         *         *         *         *         *         *         *         *
ATAGCAGAAC TTACCTGTGC AGTTGAAAGT GTTCATAAAA TGGGTTTTAT TCATAGAGAT ATTAAACCTG ATAACATTTT
  I  A  E   L  T  C  A   V  E  S   V  H  K   M  G  F  I   H  R  D   I  K  P   D  N  I  L
      2010      2020      2030      2040      2050      2060      2070      2080
         *         *         *         *         *         *         *         *
GATTGACCGT GATGGCCATA TTAAATTGAC TGACTTTGGC TTGTGCACTG GCTTCAGATG GACACATGAC TCCAAGTACT
  I  D  R   D  G  H   I  K  L  T   D  F  G   L  C  T   G  F  R  W   T  H  D   S  K  Y
      2090      2100      2110      2120      2130      2140      2150      2160
         *         *         *         *         *         *         *         *
ACCAGAGTGG GGATCACCCA CGGCAAGATA GCATGGATTT CAGTAACGAA TGGGGAGATC CTTCCAATTG TCGGTGTGGG
 Y  Q  S  G   D  H  P   R  Q  D   S  M  D  F   S  N  E   W  G  D   P  S  N  C   R  C  G
      2170      2180      2190      2200      2210      2220      2230      2240
         *         *         *         *         *         *         *         *
GACAGACTGA AGCCACTGGA GCGGAGAGCT GCTCGCCAGC ACCAGCGATG TCTAGCCCAT TCTCTGGTTG GGACTCCCAA
 D  R  L   K  P  L  E   R  R  A   A  R  Q   H  Q  R  C   L  A  H   S  L  V   G  T  P  N
```

FIG. 7D

BAD ORIGINAL

```
      2250       2260       2270       2280       2290       2300       2310       2320
         *          *          *          *          *          *          *          *
TTATATTGCA CCTGAAGTGC TACTGCGAAC AGGATATACA CAGCTGTGTG ACTGGTGGAG TGTTGGTGTT ATTCTTTGTG
 Y  I  A   P  I  V   I  L  R  T   G  Y  T   Q  L  C   D  W  W  S   V  G  V   I  L  C
      2330       2340       2350       2360       2370       2380       2390       2400
         *          *          *          *          *          *          *          *
AAATGTTGGT GGGACAACCT CCTTTCTTGG CACAAACCCC ATTAGAAACA CAAATGAAGG TTATCATCTG GCAAACTTCT
E  M  L  V   G  Q  P   P  F  L   A  Q  T  P   L  E  T   Q  M  K   V  I  I  W   Q  T  S
      2910       2420       2430       2940       2450       2960       2470       2480
         *          *          *          *          *          *          *          *
CTACACATCC CTCCTCAAGC TAAGCTGAGT CCTGAAGCCT CTGACCTCAT TATCAAACTG TGTCGAGGAC CAGAAGACCG
 L  H  I   P  P  Q  A   K  L  S   P  E  A   S  D  L  I   I  K  L   C  R  G   P  E  D  R
      2990       2500       2510       2520       2530       2540       2550       2560
         *          *          *          *          *          *          *          *
CCTCGGCAAG AACGGTGCTG ATGAGATAAA GGCTCATCCA TTTTTTAAGA CCATCGATTT CTCTAGTGAT CTGAGACAGC
 L  G  K   H  G  A   D  E  I  K   A  H  P   F  F  K   T  I  D  F   S  S  D   L  R  Q
      2570       2580       2590       2600       2610       2620       2630       2640
         *          *          *          *          *          *          *          *
AGTCTGCTTC ATACATCCCT AAAATCACGC ATCCAACAGA TACATCCAAT TTCGACCCTG TTGATCCTGA TAAATTGTGG
Q  S  A  S   Y  I  P   K  I  T   H  P  T  D   T  S  N   F  D  P   V  D  P  D   K  L  W
      2650       2660       2670       2680       2690       2700       2710       2720
         *          *          *          *          *          *          *          *
AGCGATGGCA GCGAGGAGGA AAATATCAGT GACACTCTGA GCGGATGGTA TAAAAATGGG AAGCACCCCG AGCACGCTTT
 S  D  G   S  I  L  L   N  I  S   D  T  L   S  G  W  Y   K  N  G   K  H  P   E  H  A  F
      2730       2740       2750       2760       2770       2780       2790       2800
         *          *          *          *          *          *          *          *
CTATGAGTTC ACCTTTCGGA GGTTTTTTGA TGACAATGGC TACCCATATA ATTATCCAAA GCCTATTGAG TATGAATACA
 Y  E  F   T  F  R   R  F  F  D   D  N  G   Y  P  Y   N  Y  P  K   P  I  E   Y  E  Y
```

FIG. 7E

BAD ORIGINAL

```
      2810       2820       2830       2840       2850       2860       2870       2880
         *          *          *          *          *          *          *          *
TTCATTCACA GGGCTCAGAA CAACAGTCTG ATGAAGATGA TCAACACACA AGCTCCGATG GAAACAACCG AGATCTAGTG
 I  H  S  Q   G  S  E   Q  Q  S   D  E  D  D   Q  H  T   S  S  D  G   N  N  R   D  L  V
      2890       2900       2910       2920       2930       2940       2950       2960
         *          *          *          *          *          *          *          *
TATGTTTAAT AAACTAGGAG ATCATTGTAA GAATTTGCAA GAGGCCTGAA GTGCAGGGGT TTTTGAAGTT TTGAGARAAT
 Y  V  *
      2970       2980       2990       3000       3010       3020       3030       3040
         *          *          *          *          *          *          *          *
TATGCAAATG TGACAGAGTT TGTGTGCTCT GTGTACAATA TTTTATTTTC CTAAGTTATG GGAAATTGTT TTAAAATGTT
      3050       3060       3070       3080       3090       3100       3110       3120
         *          *          *          *          *          *          *          *
AATTTATTCC ACCCTTTTAA TTCAGTAATT TAGAAAAAAT TGTTATAAGG AAAGTAAATT ATGAACTGAG TATTATAGTC
      3130       3140       3150       3160       3170       3180       3190       3200
         *          *          *          *          *          *          *          *
AATTCTTGGT ACTTAAAGTA CTTAAAAAGA GAAGCCTGGT ATCTTTTGTA TATATAATAA ATAATTTTAA AATCCCAAAA
      3210
         *
AAAAAAAAAA AAA
```

FIG. 7F

BAD ORIGINAL

```
        10         20         30         40         50         60         70         80
         *          *          *          *          *          *          *          *
ATGAGAGCCA CCCCGAAGTT TGGACCTTAT CAAAAAGCTC TCAGGGAAAT CCGATATTCC CTCCTGCCTT TTGCCAACGA
 M  R  A   I  P  K  F   G  P  Y   Q  K  A  L  R  E  I   R  Y  S   L  L  P   P  A  N  E
        90        100        110        120        130        140        150        160
         *          *          *          *          *          *          *          *
GTCAGGCACT TCGGCAGCTG CAGAGGTGAA CCGGCAGATG CTTCAGGAGT TGGTGAATGC GGCATGTGAC CAGGAGATGG
  S  G  I   S  A  A   A  E  V  N   R  Q  M  L  Q  E   L  V  N  A   A  C  D   Q  E  M
       170        180        190        200        210        220        230        240
         *          *          *          *          *          *          *          *
CTGGCAGAGC GCTCACGCAG ACGGGCAGTA GGAGTATCGA AGCTGCCTTG GAGTACATCA GTAAGATGGG CTACCTGGAC
A  G  R  A   I  I  Q   T  G  S   R  S  I  E   A  A  L   E  Y  I   S  K  M  G   Y  L  D
       250        260        270        280        290        300        310        320
         *          *          *          *          *          *          *          *
CCCAGGAATG AGCAGATTGT GCGAGTCATC AAGCAGACCT CCCCAGGAAA GGGCCTGGCG TCCACCCCGG TGACTCGGCG
 P  R  N   I  Q  I  V   R  V  I   K  Q  T   S  P  G  K   G  L  A   S  T  P   V  T  R  R
       330        340        350        360        370        380        390        400
         *          *          *          *          *          *          *          *
GCCCAGTTTC GAGGGCACAG GGGAAGCACT CCCATCCTAC CACCAGCTGG GTGGTGCAAA CTACGAGGGC CCCGCCGCAC
  P  S  F   I  G  I   G  F  A  L   P  S  Y   H  Q  L   G  G  A  N   Y  E  G   P  A  A
       410        420        430        440        450        460        470        480
         *          *          *          *          *          *          *          *
TGGAGGAGAT GCCGCGGCAA TATTTAGACT TTCTCTTCCC TGGAGCCGGA GCCGGCACCC ACGGTGCCCA GGCTCACCAG
L  E  E  M   P  R  Q   Y  L  D   F  L  F  P   G  A  G   A  G  T   H  G  A  Q   A  H  Q
       490        500        510        520        530        540        550        560
         *          *          *          *          *          *          *          *
CATCCTCCCA AAGGGTACAG CACAGCAGTA GAGCCAAGTG CGCACTTTCC GGGCACACAC TATGGTCGTG GTCATCTACT
 H  P  P   K  G  Y  S   T  A  V   E  P  S   A  H  F  P   G  T  H   Y  G  R   G  H  L  L
```

FIG. 8A

```
       570        580        590        600        610        620        630        640
         *          *          *          *          *          *          *          *
ATCGGAGCAG TCTGGGTATG GGGTGCAGCG CAGTTCCTCC TTCCAGAACA AGACGCCACC AGATGCCTAT TCCAGCATGG
 S  E  Q   S  G  Y   G  V  Q  R   S  S  S   F  Q  N   K  T  P  P   D  A  Y   S  S  M
       650        660        670        680        690        700        710        720
         *          *          *          *          *          *          *          *
CCAAGGCCCA GGGTGGCCCT CCCGCCAGCC TCACCTTTCC TGGCCATGCT GGGCTGTACA CTGCCTCGCA CCACAAGCCG
A  K  A  Q   G  G  P   P  A  S  L  T  F  P   A  H  A   G  L  Y   T  A  S  H   H  K  P
       730        740        750        760        770        780        790        800
         *          *          *          *          *          *          *          *
GCGGCTACCC CACCTGGGGC CCACCCATTA CATGTGTTGG GCACCCGGGG TCCCACGTTT ACTGGCGAAA GCTCTGCACA
 A  A  I   P  P  G  A   H  P  L   H  V  L   G  T  R  G   P  T  F   T  G  E   S  S  A  Q
       810        820        830        840        850        860        870        880
         *          *          *          *          *          *          *          *
GGCTGTGCTG GCACCGTCCA GGAACAGCCT CAATGCTGAC TTGTACGAGC TGGGCTCCAC GGTGCCCTGG TCTGCAGCTC
 A  V  L   A  P  S   R  N  S  L   N  A  D   L  Y  E   L  G  S  T   V  P  W   S  A  A
       890        900        910        920        930        940        950        960
         *          *          *          *          *          *          *          *
CACTGGCACG CCGCGACTCG CTGCAGAAGC AGGGTCTAGA AGCCTCGCGG CCGCATGTGG CTTTTCGGGC TGGCCCCAGC
P  L  A  R   R  D  S   L  Q  K   Q  G  L  E   A  S  R   P  H  V   A  F  R  A   G  P  S
       970        980        990       1000       1010       1020       1030       1040
         *          *          *          *          *          *          *          *
AGGACCAACT CCTTCAACAA CCCACAACCT GAGCCCTCAC TGCCCGCCCC CAACACGGTC ACCGCCGTGA CGGCCGCACA
 R  T  N   S  F  N  N   P  Q  P   E  P  S   L  P  A  P   N  T  V   T  A  V   T  A  A  H
      1050       1060       1070       1080       1090       1100       1110       1120
         *          *          *          *          *          *          *          *
CATCCTTCAC CCTGTGAAGA GCGTGCGTGT GCTGCGGCCC GAGCCCCAGA CAGCCGTGGG GCCCTCGCAC CCCGCCTGGG
 I  I  H   P  V  K   S  V  R  V   L  R  P   E  P  Q   T  A  V  G   P  S  H   P  A  W
```

FIG. 8B

```
      1130       1140       1150       1160       1170       1180       1190       1200
         *          *          *          *          *          *          *          *
TGGCTGCGCC CACAGCACCT GCCACTGAGA GCCTGGAGAC GAAGGAGGGC AGCGCAGGCC CACACCCGCT GGATGTGGAC
 A  A  P   T  A  P   A  T  E   S  L  E  T   K  E  G   S  A  G   P  H  P  L   D  V  D
      1210       1220       1230       1240       1250       1260       1270       1280
         *          *          *          *          *          *          *          *
TATGGCGGCT CCGAGCGCAG GTGCCCACCG CCTCCGTATC CAAAGCACTT GCTGCTGCCC AGTAAGTCTG AGCAGTACAG
 Y  G  G   S  E  R  R   C  P  P   P  P  Y   P  K  H  L   L  L  P   S  K  S   E  Q  Y  S
      1290       1300       1310       1320       1330       1340       1350       1360
         *          *          *          *          *          *          *          *
CGTGGACCTG GACAGCCTGT GCACCAGTGT GCAGCAGAGT CTGCGAGGGG GCACTGATCT AGACGGGAGT GACAAGAGCC
 V  D  L   D  S  L   C  T  S  V   Q  Q  S   L  R  G   G  T  D  L   D  G  S   D  K  S
      1370       1380       1390       1400       1410       1420       1430       1440
         *          *          *          *          *          *          *          *
ACAAAGGTGC GAAGGGAGAC AAAGCTGGCA GAGACAAAAA GCAGATTCAG ACCTCCCCGG TGCCTGTCCG CAAGAATAGC
H  K  G  A   K  G  D   K  A  G   R  D  K  K   Q  I  Q   T  S  P   V  P  V  R   K  N  S
      1450       1460       1470       1480       1490       1500       1510       1520
         *          *          *          *          *          *          *          *
AGAGATGAAG AGAAGAGAGA GTCTCGCATC AAGAGTTACT CCCCTTATGC CTTCAAATTC TTCATGGAGC AACACGTGGA
 R  D  E   E  K  R  E   S  R  I   K  S  Y   S  P  Y  A   F  K  F   F  M  E   Q  H  V  E
      1530       1540       1550       1560       1570       1580       1590       1600
         *          *          *          *          *          *          *          *
GAATGTCATC AAAACCTACC AGCAGAAGGT CAGCCGGAGG CTACAGCTGG AGCAGGAAAT GGCCAAAGCT GGGCTCTGTG
 N  V  I   K  T  Y   Q  Q  K  V   S  R  R   L  Q  L   E  Q  E  M   A  K  A   G  L  C
      1610       1620       1630       1640       1650       1660       1670       1680
         *          *          *          *          *          *          *          *
AGGCCGAGCA GGAGCAGATG AGGAAGATCC TCTACCAGAA GGAGTCTAAC TACAACCGGC TGAAGAGGGC CAAGATGGAC
E  A  E  Q   E  Q  M   R  K  I   L  Y  Q  K   E  S  N   Y  N  R   L  K  R  A   K  M  D
```

FIG. 8C

BAD ORIGINAL

```
      1690       1700       1710       1720       1730       1740       1750       1760
         *          *          *          *          *          *          *          *
AAGTCCATGT TTGTGAAAAT CAAGACTCTA GGCATCGGTG CCTTTGGGGA AGTGTGCCTC GCTTGTAAGC TGGACACTCA
 K  S  H   F  V  K  I   K  T  L   G  I  G   A  F  G  E   V  C  L   A  C  K   L  D  T  H
      1770       1780       1790       1800       1810       1820       1830       1840
         *          *          *          *          *          *          *          *
CGCTCTGTAC GCCATGAAGA CTCTCAGGAA GAAGGATGTC CTGAACCGGA ATCAAGTGGC CCATGTCAAG GCTGAGAGGG
  A  L  Y   A  M  K   T  L  R  K   K  D  V   L  N  R   N  Q  V  A   H  V  K   A  E  R
      1850       1860       1870       1880       1890       1900       1910       1920
         *          *          *          *          *          *          *          *
ACATCCTGGC TGAAGCAGAC AATGAGTGGG TGGTCAAACT CTACTACTCC TTCCAGGACA AGGACAGCCT GTACTTTGTG
D  I  L  A   T  A  D   N  E  W   V  V  K  L   Y  Y  S   F  Q  D   K  D  S  L   Y  F  V
      1930       1940       1950       1960       1970       1980       1990       2000
         *          *          *          *          *          *          *          *
ATGGACTACA TACCAGGCGG GGATATGATG AGCCTGCTGA TCAGGATGGA GGTCTTCCCT GAGCACCTGG CCCGCTTCTA
 M  D  Y   I  P  G  G   D  M  M   S  L  L   I  R  M  E   V  F  P   E  H  L   A  R  F  Y
      2010       2020       2030       2090       2050       2060       2070       2080
         *          *          *          *          *          *          *          *
CATTGCAGAG TTGACCCTGG CCATTGAAAG TGTCCACAAG ATGGGCTTTA TCCACCGGGA CATCAAGCCT GACAACATAC
  I  A  L   I  T  L   A  I  E  S   V  H  K   M  G  F   I  H  R  D   I  K  P   D  N  I
      2090       2100       2110       2120       2130       2140       2150       2160
         *          *          *          *          *          *          *          *
TCATCGACCT GGATGGTCAT ATTAAGCTGA CAGATTTTGG CCTCTGCACT GGATTCAGGT GGACTCACAA TTCCAAGTAC
L  I  D  L   D  G  H   I  K  L   T  D  F  G   L  C  T   G  F  R   W  T  H   N  S  K  Y
      2170       2180       2190       2200       2210       2220       2230       2240
         *          *          *          *          *          *          *          *
TACCAGAAAG GGAACCACAT GAGACAGGAC AGCATGGAGC CCGGTGACCT CTGGGACGAT GTTTCCAACT GTCGCTGTGG
 Y  Q  E   G  N  H  M   R  Q  D   S  M  E   P  G  D  L   W  D  D   V  S  N   C  R  C  G
```

FIG. 8D

BAD ORIGINAL

```
      2250       2260       2270       2280       2290       2300       2310       2320
         *          *          *          *          *          *          *          *
AGACAGGTTA AAGACCCTGG AGCAGAGGGC GCAGAAGCAG CACCAGAGGT GCCTGGCACA TTCTCTTGTC GGGACACCAA
 D  R  L   K  I  L   F  Q  R  A   Q  K  Q   H  Q  R   C  L  A  H   S  L  V   G  T  P
      2330       2340       2350       2360       2370       2380       2390       2400
         *          *          *          *          *          *          *          *
ATTACATCGC TCCGGAGGTG CTTCTCCGCA AAGGGTACAC GCAGCTCTGT GACTGGTGGA GCGTCGGTGT GATTCTCTTT
N  Y  I  A   P  E  V   L  L  R   K  G  Y  T   Q  L  C   D  W  W   S  V  G  V   I  L  F
      2410       2420       2430       2440       2450       2460       2470       2480
         *          *          *          *          *          *          *          *
GAGATGCTGG TTGGGCAGCC GCCTTTCTTG GCCCCCACCC CCACAGAGAC GCAGCTGAAG GTGATCAACT GGGAGAGCAC
 E  M  L   V  G  Q  P   P  F  L   A  P  T   P  T  E  T   Q  L  K   V  I  N   W  E  S  T
      2490       2500       2510       2520       2530       2540       2550       2560
         *          *          *          *          *          *          *          *
GCTGCATATC CCTACGCAGG TGAGGCTCAG CGCTGAGGCC CGAGACCTCA TCACGAAGCT GTGCTGCGCG GCTGACTGCC
 L  H  I   P  I  Q   V  R  L  S   A  E  A   R  D  L   I  T  K  L   C  C  A   A  D  C
      2570       2580       2590       2600       2610       2620       2630       2640
         *          *          *          *          *          *          *          *
GCCTGGGCAG GGATGGGGCA GATGACCTCA AGGCACACCC GTTCTTCAAC ACCATCGACT TTTCCCGTGA CATCCGAAAG
R  L  G  R   D  G  A   D  D  L   K  A  H  P   F  F  N   T  I  D   F  S  R  D   I  R  K
      2650       2660       2670       2680       2690       2700       2710       2720
         *          *          *          *          *          *          *          *
CAGGCTGCAC CCTACGTCCC CACCATCAGC CACCCCATGG ACACCTCCAA TTTTGACCCG GTGGATGAAG AAAGCCCCTG
 Q  A  A   P  Y  V  P   T  I  S   H  P  M   D  T  S  N   F  D  P   V  D  E   E  S  P  W
      2730       2740       2750       2760       2770       2780       2790       2800
         *          *          *          *          *          *          *          *
GCACGAGGCC AGCGGAGAGA GCGCCAAGGC CTGGGACACG CTGGCCTCCC CCAGCAGCAA GCATCCAGAG CACGCCTTCT
 H  I  A   S  G  E   S  A  K  A   W  D  T   L  A  S   P  S  S  K   H  P  E   H  A  F
```

FIG. 8E

BAD ORIGINAL

```
      2810       2820       2830       2840       2850       2860       2870       2880
         *          *          *          *          *          *          *          *
ATGAGTTCAC CTTCCGCAGG TTCTTCGATG ACAACGGCTA TCCCTTCCGG TGCCCGAAGC CCTCAGAGCC CGCAGAGAGT
 Y  E  F  T   F  R  R  F  F  D   D  N  G  Y   P  F  R  C  P  K   P  S  E  P   A  E  S
      2890       2900       2910       2920       2930       2940       2950       2960
         *          *          *          *          *          *          *          *
GCAGACCCAG GGGATGCGGA CTTGGAAGGT GCGGCCGAGG GCTGCCAGCC GGTGTACGTG TAAGCCTCAG TTAACCACAA
 A  D  P   G  D  A  D   L  E  G   A  A  E   G  C  Q  P   V  Y  V   *
      2970       2980       2990       3000       3010       3020       3030       3040
         *          *          *          *          *          *          *          *
CTCGAGGAAA CCCAAAATGA GATTTCTTTT CAGAAGACAA ACTCAAGCTT AGGAATCCTT CATTTTTAGT TCTGGTAAAT
      3050       3060       3070       3080       3090       3100       3110       3120
         *          *          *          *          *          *          *          *
GGGCAACAGG AAGAGTCAAC ATGATTTCAA ATTAGCCCTC TGAGGACCTT CACTGCATTA AAACAGTATT TTTTAAAAAA
      3130       3140       3150
         *          *          *
TTAGTACAGT ATGGAAAGAG CACTTATTTT GGGGG
```

FIG. 8F

BAD ORIGINAL

```
        10         20         30         40         50         60         70         80
         *          *          *          *          *          *          *          *
ACCTTTGGGT TGCTGGGACG GACTCTGGCC GCCTCAGCGT CCGCCCTCAG GCCCGTGGCC GCTGTCCAGG AGCTCTGCTC
        90        100        110        120        130        140        150        160
         *          *          *          *          *          *          *          *
TCCCCTCCAG AGTTAATTAT TTATATTGTA AAGAATTTTA ACAGTCCTGG GGACTTCCTT GAAGGATCAT TTTCACTTTT
       170        180        190        200        210        220        230        240
         *          *          *          *          *          *          *          *
GCTCAGAAGA AAGCTCTGGA TCTATCAAAT AAAGAAGTCC TTCGTGTGGG CTACATATAT AGATGTTTTC ATGAAGAGGA
                                                                               M  K  R
       250        260        270        280        290        300        310        320
         *          *          *          *          *          *          *          *
GTGAAAAGCC AGAAGGATAT AGACAAATGA GGCCTAAGAC CTTTCCTGCC AGTAACTATA CTGTCAGTAG CCGGCAAATG
 S  E  K  P   I  G  Y   R  Q  M   R  P  K  T   F  P  A   S  N  Y   T  V  S  S   R  Q  M
       330        340        350        360        370        380        390        400
         *          *          *          *          *          *          *          *
TTACAAGAAA TTCGGGAATC CCTTAGGAAT TTATCTAAAC CATCTGATGC TGCTAAGGCT GAGCATAACA TGAGTAAAAT
 L  Q  L   I  R  L  S   L  R  N   L  S  K   P  S  D  A   A  K  A   E  H  N   M  S  K  M
       410        420        430        440        450        460        470        480
         *          *          *          *          *          *          *          *
GTCAACCGAA GATCCTCGAC AAGTCAGAAA TCCACCCAAA TTTGGGACGC ATCATAAAGC CTTGCAGGAA ATTCGAAACT
   S  I  E   D  P  R   Q  V  R  N   P  P  K   F  G  T   H  H  K  A   L  Q  E   I  R  N
       490        500        510        520        530        590        550        560
         *          *          *          *          *          *          *          *
CTCTGCTTCC ATTTGCAAAT GAAACAAATT CTTCTCGGAG TACTTCAGAA GTTAATCCAC AAATGCTTCA AGACTTGCAA
 S  L  I  P   F  A  N   E  T  N   S  S  R  S   T  S  E   V  N  P   Q  M  L  Q   D  L  Q
```

FIG. 9A

BAD ORIGINAL

```
       570        580        590        600        610        620        630        640
         *          *          *          *          *          *          *          *
GCTGCTGGAT TTGATGAGGA TATGGTTATA CAAGCTCTTC AGAAAACTAA CAACAGAAGT ATAGAAGCAG CAATTGAATT
 A  A  G   I  D  E  D   M  V  I   Q  A  L   Q  K  T  N   N  R  S   I  E  A   A  I  E  F
       650        660        670        680        690        700        710        720
         *          *          *          *          *          *          *          *
CATTAGTAAA ATGAGTTACC AAGATCCTCG ACGAGAGCAG ATGGCTGCAG CAGCTGCCAG ACCTATTAAT GCCAGCATGA
 I  S  K   M  S  Y   Q  D  P  R   R  E  Q   M  A  A   A  A  A  R   P  I  N   A  S  M
       730        740        750        760        770        780        790        800
         *          *          *          *          *          *          *          *
AACCAGGGAA TGTGCAGCAA TCAGTTAACC GCAAACAGAG CTGGAAAGGT TCTAAAGAAT CCTTAGTTCC TCAGAGGCAT
K  P  G  N   V  Q  Q   S  V  N   R  K  Q  S   W  K  G   S  K  E   S  L  V  P   Q  R  H
       810        820        830        840        850        860        870        880
         *          *          *          *          *          *          *          *
GGCCCGCCAC TAGGAGAAAG TGTGGCCTAT CATTCTGAGA GTCCCAACTC ACAGACAGAT GTAGGAAGAC CTTTGTCTGG
 G  P  P   I  G  E  S   V  A  Y   H  S  E   S  P  N  S   Q  T  D   V  G  R   P  L  S  G
       890        900        910        920        930        940        950        960
         *          *          *          *          *          *          *          *
ATCTGGTATA TCAGCATTTG TTCAAGCTCA CCCTAGCAAC GGACAGAGAG TGAACCCCCC ACCACCACCT CAAGTAAGGA
 S  G  I   S  A  F   V  Q  A  H   P  S  N   G  Q  R   V  N  P  P   P  P  P   Q  V  R
       970        980        990       1000       1010       1020       1030       1040
         *          *          *          *          *          *          *          *
GTGTTACTCC TCCACCACCT CCAAGAGGCC AGACTCCCCC TCCAAGAGGT ACAACTCCAC CTCCCCCTTC ATGGGAACCA
S  V  I  P   P  P  P   P  R  G   Q  T  P  P   P  R  G   T  T  P   P  P  P  S   W  E  P
      1050       1060       1070       1080       1090       1100       1110       1120
         *          *          *          *          *          *          *          *
AACTCTCAAA CAAAGCGCTA TTCTGGAAAC ATGGAATACG TAATCTCCCG AATCTCTCCT GTCCCACCTG GGGCATGGCA
 N  S  Q   I  K  R  Y   S  G  N   M  E  Y   V  I  S  R   I  S  P   V  P  P   G  A  W  Q
```

FIG. 9B

BAD ORIGINAL

```
      1130       1140       1150       1160       1170       1180       1190       1200
         *          *          *          *          *          *          *          *
AGAGGGCTAT CCTCCACCAC CTCTCAACAC TTCCCCCATG AATCCTCCTA ATCAAGGACA GAGAGGCATT AGTTCTGTTC
  E  G  Y   P  P  P   P  L  N  T   S  P  M   N  P  P   N  Q  G  Q   R  G  I   S  S  V
      1210       1220       1230       1240       1250       1260       1270       1280
         *          *          *          *          *          *          *          *
CTGTTGGCAG ACAACCAATC ATCATGCAGA GTTCTAGCAA ATTTAACTTT CCATCAGGGA GACCTGGAAT GCAGAATGGT
P  V  G  R   Q  P  I   I  M  Q   S  S  S  K   F  N  F   P  S  G   R  P  G  M   Q  N  G
      1290       1300       1310       1320       1330       1340       1350       1360
         *          *          *          *          *          *          *          *
ACTGGACAAA CTGATTTCAT GATACACCAA AATGTTGTCC CTGCTGGCAC TGTGAATCGG CAGCCACCAC CTCCATATCC
 T  G  Q   T  D  F  M   I  H  Q   N  V  V   P  A  G  T   V  N  R   Q  P  P   P  P  Y  P
      1370       1380       1390       1400       1410       1420       1430       1440
         *          *          *          *          *          *          *          *
TCTGACAGCA GCTAATGGAC AAAGCCCTTC TGCTTTACAA ACAGGGGGAT CTGCTGCTCC TTCGTCATAT ACAAATGGAA
  L  T  A   A  N  G   Q  S  P  S   A  L  Q   T  G  G   S  A  A  P   S  S  Y   T  N  G
      1450       1460       1470       1480       1490       1500       1510       1520
         *          *          *          *          *          *          *          *
GTATTCCTCA GTCTATGATG GTGCCAAACA GAAATAGTCA TAACATGGAA CTATATAACA TTAGTGTACC TGGACTGCAA
S  I  P  Q   S  M  M   V  P  N   R  N  S  H   N  M  E  L  Y  N  I   S  V  P   G  L  Q
      1530       1540       1550       1560       1570       1580       1590       1600
         *          *          *          *          *          *          *          *
ACAAATTGGC CTCAGTCATC TTCTGCTCCA GCCCAGTCAT CCCCGAGCAG TGGGCATGAA ATCCCTACAT GGCAACCTAA
 T  N  W   P  Q  S  S   S  A  P   A  Q  S   S  P  S  S   G  H  E   I  P  T   W  Q  P  N
      1610       1620       1630       1640       1650       1660       1670       1680
         *          *          *          *          *          *          *          *
CATACCAGTG AGGTCAAATT CTTTTAATAA CCCATTAGGA AATAGAGCAA GTCACTCTGC TAATTCTCAG CCTTCTGCTA
 I  P  V   R  S  N   S  F  N  N   P  L  G   N  R  A   S  H  S  A   N  S  Q   P  S  A
```

FIG. 9C

BAD ORIGINAL

```
      1690       1700       1710       1720       1730       1740       1750       1760
         *          *          *          *          *          *          *          *
CAACAGTCAC TGCAATTACA CCAGCTCCTA TTCAACAGCC TGTGAAAAGT ATGCGTGTAT TAAAACCAGA GCTACAGACT
T  T  V  I   A  I  T   P  A  P   I  Q  Q  P   V  K  S   M  R  V   L  K  P  E   L  Q  T
      1770       1780       1790       1800       1810       1820       1830       1840
         *          *          *          *          *          *          *          *
GCTTTAGCAC CTACACACCC TTCTTGGATA CCACAGCCAA TTCAAACTGT TCAACCCAGT CCTTTTCCTG AGGGAACCGC
 A  L  A   P  T  H  P   S  W  I   P  Q  P   I  Q  T  V   Q  P  S   P  F  P   E  G  T  A
      1850       1860       1870       1880       1890       1900       1910       1920
         *          *          *          *          *          *          *          *
TTCAAATGTG ACTGTGATGC CACCTGTTGC TGAAGCTCCA AACTATCAAG GACCACCACC ACCCTACCCA AAACATCTGC
  S  N  V   I  V  M   P  P  V  A   E  A  P   N  Y  Q   G  P  P  P   P  Y  P   K  H  L
      1930       1940       1950       1960       1970       1980       1990       2000
         *          *          *          *          *          *          *          *
TGCACCAAAA CCCATCTGTT CCTCCATACG AGTCAATCAG TAAGCCTAGC AAAGAGGATC AGCCAAGCTT GCCCAAGGAA
I  H  Q  H   P  S  V   P  P  Y   E  S  I  S   K  P  S   K  E  D   Q  P  S  L   P  K  E
      2010       2020       2030       2040       2050       2060       2070       2080
         *          *          *          *          *          *          *          *
GATGAGAGTG AAAAGAGTTA TGAAAATGTT GATAGTGGGG ATAAAGAAAA GAAACAGATT ACAACTTCAC CTATTACTGT
 D  F  S   I  K  S  Y   E  N  V   D  S  G   D  K  E  K   K  Q  I   T  T  S   P  I  T  V
      2090       2100       2110       2120       2130       2140       2150       2160
         *          *          *          *          *          *          *          *
TAGGAAAAAC AAGAAAGATG AAGAGCGAAG GGAATCTCGT ATTCAAAGTT ATTCTCCTCA AGCATTTAAA TTCTTTATGG
  R  K  N   K  K  D   E  E  R  R   E  S  R   I  Q  S   Y  S  P  Q   A  F  K   F  F  M
      2170       2180       2190       2200       2210       2220       2230       2240
         *          *          *          *          *          *          *          *
AGCAACATGT AGAAAATGTA CTCAAATCTC ATCAGCAGCG TCTACATCGT AAAAAACAAT TAGAGAATGA AATGATGCGG
I  Q  H  V   I  H  V   I  K  S   H  Q  Q  R   L  H  R   K  K  Q   L  F  N  E   M  M  R
```

FIG. 9D

BAD ORIGINAL

```
      2250       2260       2270       2280       2290       2300       2310       2320
         *          *          *          *          *          *          *          *
GTTGGATTAT CTCAAGATGC CCAGGATCAA ATGAGAAAGA TGCTTTGCCA AAAAGAATCT AATTACATCC GTCTTAAAAG
 V  G  L   S  Q  D  A   Q  D  Q   M  R  K   M  L  C  Q   K  E  S   N  Y  I   R  L  K  R
      2330       2340       2350       2360       2370       2380       2390       2400
         *          *          *          *          *          *          *          *
GGCTAAAATG GACAAGTCTA TGTTTGTGAA GATAAAGACA CTAGGAATAG GAGCATTTGG TGAAGTCTGT CTAGCAAGAA
  A  K  M   D  K  S   M  F  V  K   I  K  T   L  G  I   G  A  F  G   E  V  C   L  A  R
      2410       2420       2430       2440       2950       2460       2470       2480
         *          *          *          *          *          *          *          *
AAGTAGATAC TAAGGCTTTG TATGCAACAA AAACTCTTCG AAAGAAAGAT GTTCTTCTTC GAAATCAAGT CGCTCATGTT
K  V  D  T   K  A  L   Y  A  T   K  T  L  R   K  K  D   V  L  L   R  N  Q  V   A  H  V
      2490       2500       2510       2520       2530       2540       2550       2560
         *          *          *          *          *          *          *          *
AAGGCTGAGA GAGATATCCT GGCTGAAGCT GACAATGAAT GGGTAGTTCG TCTATATTAT TCATTCCAAG ATAAGGACAA
 K  A  E   R  D  I  L   A  E  A   D  N  E   W  V  V  R   L  Y  Y   S  F  Q   D  K  D  N
      2570       2580       2590       2600       2610       2620       2630       2640
         *          *          *          *          *          *          *          *
TTTATACTTT GTAATGGACT ACATTCCTGG GGGTGATATG ATGAGCCTAT TAATTAGAAT GGGCATCTTT CCAGAAAGTC
 L  Y  I   V  M  D   Y  I  P  G   G  D  M   M  S  L   L  I  R  M   G  I  F   P  E  S
      2650       2660       2670       2680       2690       2700       2710       2720
         *          *          *          *          *          *          *          *
TGGCACGATT CTACATAGCA GAACTTACCT GTGCAGTTGA AAGTGTTCAT AAAATGGGTT TTATTCATAG AGATATTAAA
L  A  R  I   Y  I  A   E  L  T   C  A  V  E   S  V  H   K  M  G   F  I  H  R   D  I  K
      2730       2740       2750       2760       2770       2780       2790       2800
         *          *          *          *          *          *          *          *
CCTGATAATA TTTTGATTGA TCGTGATGGT CATATTAAAT TGACTGACTT TGGCCTCTGC ACTGGCTTCA GATGGACACA
 P  D  N   I  I  I  D   R  D  G   H  I  K   L  T  D  F   G  L  C   T  G  F  R   W  T  H
```

FIG. 9E

BAD ORIGINAL

```
      2810       2820       2830       2840       2850       2860       2870       2880
         *          *          *          *          *          *          *          *
CGATTCTAAG TACTATCAGA GTGGTGACCA TCCACGGCAA GATAGCATGG ATTTCAGTAA TGAATGGGGG GATCCCTCAA
 D  S  K   Y  Y  Q   S  G  D  H   P  R  Q   D  S  M   D  F  S  N   E  W  G   D  P  S
      2890       2900       2910       2920       2930       2940       2950       2960
         *          *          *          *          *          *          *          *
GCTGTCGATG TGGAGACAGA CTGAAGCCAT TAGAGCGGAG AGCTGCACGC CAGCACCAGC GATGTCTAGC ACATTCTTTG
S  C  R  C   G  D  R   L  K  P   L  E  R  R   A  A  R   Q  H  Q   R  C  L  A   H  S  L
      2970       2980       2990       3000       3010       3020       3030       3040
         *          *          *          *          *          *          *          *
GTTGGGACTC CCAATTATAT TGCACCTGAA GTGTTGCTAC GAACAGGATA CACACAGTTG TGTGATTGGT GGAGTGTTGG
 V  G  T   P  N  Y  I   A  P  E   V  L  L   R  T  G  Y   T  Q  L   C  D  W   W  S  V  G
      3050       3060       3070       3080       3090       3100       3110       3120
         *          *          *          *          *          *          *          *
TGTTATTCTT TTTGAAATGT TGGTGGGACA ACCTCCTTTC TTGGCACAAA CACCATTAGA AACACAAATG AAGGTTATCA
 V  I  L   F  E  M   L  V  G  Q   P  P  F   L  A  Q   T  P  L  E   T  Q  M   K  V  I
      3130       3140       3150       3160       3170       3180       3190       3200
         *          *          *          *          *          *          *          *
ACTGGCAAAC ATCTCTTCAC ATTCCACCAC AAGCTAAACT CAGTCCTGAA GCTTCTGATC TTATTATTAA ACTTTGCCGA
N  W  Q  T   S  L  H   I  P  P   Q  A  K  L   S  P  E   A  S  D   L  I  I  K   L  C  R
      3210       3220       3230       3240       3250       3260       3270       3280
         *          *          *          *          *          *          *          *
GGACCCGAAG ATCGCTTAGG CAAGAATGGT GCTGATGAAA TAAAAGCTCA TCCATTTTTT AAAACAATTG ACTTCTCCAG
 G  P  E   D  R  L  G   K  N  G   A  D  E   I  K  A  H   P  F  F   K  T  I   D  F  S  S
      3290       3300       3310       3320       3330       3340       3350       3360
         *          *          *          *          *          *          *          *
TGACCTGAGA CAGCAGTCTG CTTCATACAT TCCTAAAATC ACACACCCAA CAGATACATC AAATTTTGAT CCTGTTGATC
 D  I  R   Q  Q  S   A  S  Y  I   P  K  I   T  H  P   T  D  T  S   N  F  D   P  V  D
```

FIG.9F

BAD ORIGINAL

```
      3370      3380      3390      3400      3410      3420      3430      3440
         *         *         *         *         *         *         *         *
CTGATAAATT ATGGAGTGAT GATAACGAGG AAGAAAATGT AAATGACACT CTCAATGGAT GGTATAAAAA TGGAAAGCAT
P  D  K  I   W  S  D   D  N  E  E  E  N  V  N  D  T   L  N  G  W   Y  K  N   G  K  H
      3450      3460      3470      3480      3490      3500      3510      3520
         *         *         *         *         *         *         *         *
CCTGAACATG CATTCTATGA ATTTACCTTC CGAAGGTTTT TTGATGACAA TGGCTACCCA TATAATTATC CGAAGCCTAT
 P  E  H   A  F  Y  E   F  T  F   R  R  F   F  D  D  N   G  Y  P   Y  N  Y   P  K  P  I
      3530      3540      3550      3560      3570      3580      3590      3600
         *         *         *         *         *         *         *         *
TGAATATGAA TACATTAATT CACAAGGCTC AGAGCAGCAG TCGGATGAAG ATGATCAAAA CACAGGCTCA GAGATTAAAA
  E  Y  E   Y  I  N   S  Q  G  S   E  Q  Q   S  D  E   D  D  Q  N   T  G  S   E  I  K
      3610      3620      3630      3640      3650      3660      3670      3680
         *         *         *         *         *         *         *         *
ATCGCGATCT AGTATATGTT TAACACACTA GTAAATAAAT GTAATGAGGA TTTGTAAAAG GGCCTGAAAT GCGAGGTGTG
N  R  D  L   V  Y  V   *
      3690      3700      3710      3720      3730      3740      3750      3760
         *         *         *         *         *         *         *         *
TTGAGGTTCT GAGAGTAAAA TTATGCAAAT ATGACAGAGC TATATATGTG TGCTCTGTGT ACAATATTTT ATTTTCCTAA
      3770      3780      3790      3800      3810      3820      3830      3840
         *         *         *         *         *         *         *         *
ATTATGGGAA ATCCTTTTAA AATGTTAATT TATTCCAGCC GTTTAAATCA GTATTTAGAA AAAAATTGTT ATAAGGAAAG
      3850      3860      3870      3880      3890      3900      3910      3920
         *         *         *         *         *         *         *         *
TAAATTATGA ACTGAATATT ATAGTCAGTT CTTGGTACTT AAAGTACTTA AAATAAGTAG TGCTTTGTTT AAAAGGAGAA
      3930      3940      3950      3960      3970      3980
         *         *         *         *         *         *
ACCTGGTATC TATTTGTATA TATGCTAAAT AATTTTAAAA TACAAGAGTT TTTGAAATTT TTTT
```

FIG. 9G

BAD ORIGINAL

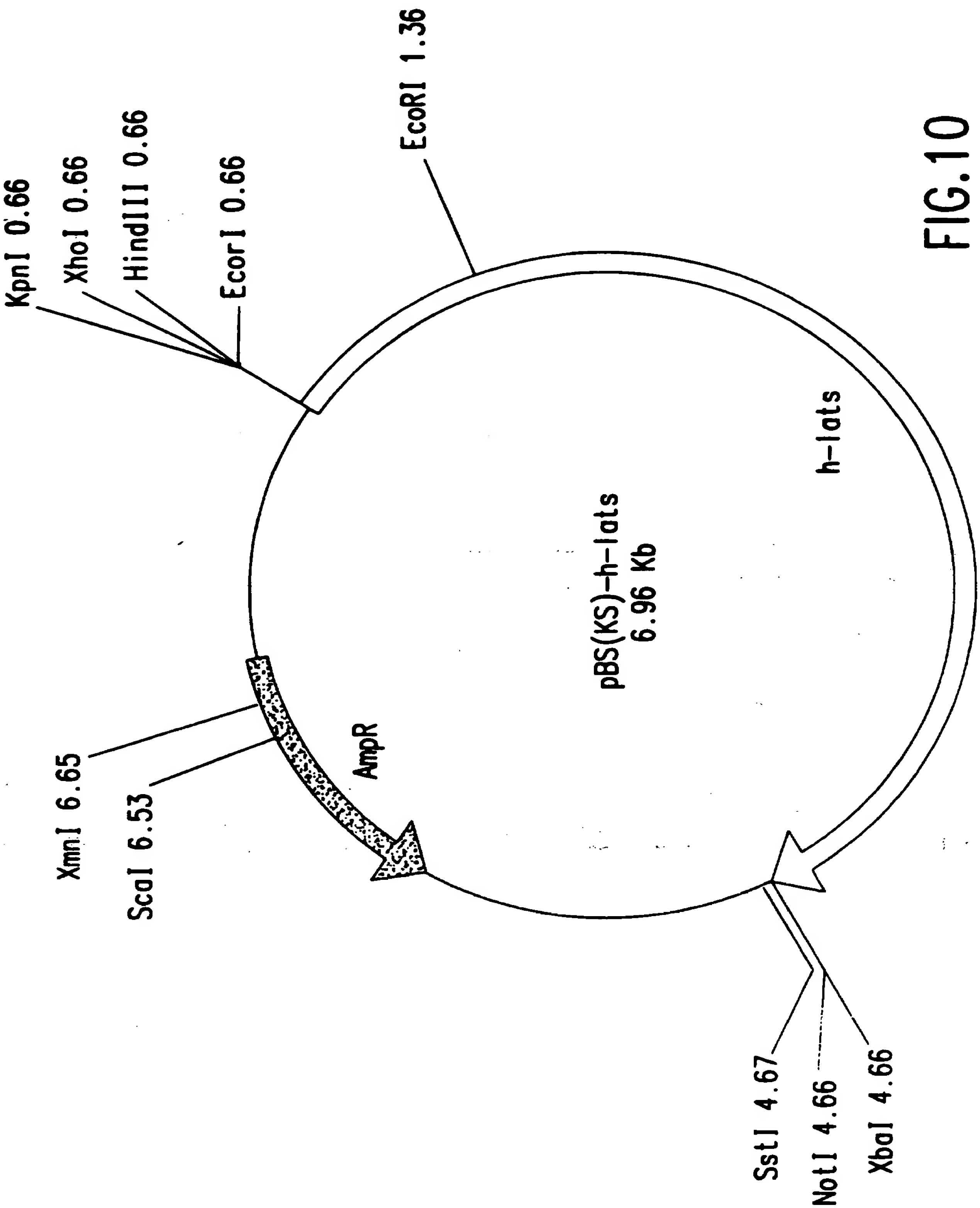

KpnI 0.66
XhoI 0.66
HindIII 0.66
EcorI 0.66
EcoRI 1.36
h-lats
pBS(KS)-h-lats
6.96 Kb
AmpR
XmnI 6.65
ScaI 6.53
SstI 4.67
NotI 4.66
XbaI 4.66

FIG.10

```
hAIS MKRSEKPEGYRQMRPKTFPASNYTVSSRQMLQEIRESLRNLSKPSDAAKAEHNMSKMSTEDPRQVRNPPK 70

hAIS LGIHHKALQFIRNSLLPFANETNSSRSTSEVNPQMLQDLQAAGFDEDMVIQALQRTNNRSIEAAIEFISK 140

hAIS MSYQDPRREQMAAAAARPINASMKPGNVQQSVNRKQSWKGSKESLVPQRHGPPLGESVAYHSESPNSQTD 210
mAIS                             ..h.i..................s..n.v.r........a. 45

hAIS VGRPLSGSGISAFVQAHPSNGQRVNPPPPPQVRSVTPPPPPRGQTPPPRGTTPPPPSWEPNSQTKRYSGN 280
mAIS ..........a..a.........................................s......... 117

hAIS MEYVISRISPVPPGAWQEGYPPPPLNTSPMNPPNQGQRGISSVPVGRQPIIMQSSSRFNFPSGRPGMQNG 350
mAIS ......................t.......s.a..a..............t.....tP....v... 187

hAIS IGQIDFMIHQNVVPAGTVNRQPPPPYPLTAANGQSPSALQTGGSAAPSSYTNGSIPQSMMVPNRNSHNME 420
mAIS q..s..iv.....t.s.t..........p...........a....p.fa..nv.............. 256

hAIS LYNISVPGLQINWPQSSSAPAQSSPSSGHEIPTWQPNIPVRSNSFNNPLGNRASHSANSQPSATTVTAIT 490
mAIS ....n.....a.............g.....................s.................. 326

hAIS PAPIQQPVKSMRVLKPELQTALAPTHPSWIPQPIQTVQPSPFPEGTASNVTVMPPVAEAPNYQGPPPPYP 560
mAIS ..........................m...v.....t..s.....s.p.i.......s......... 396

hAIS KHLLHQNPSVPPYESISKPSKEDQPSLPKEDESEKSYENVDSGDKEKKQITTSPITVRKNKKDEERRESR 630
mAIS ..............v...c.de........d.....adsg........................... 466

hAIS IQSYSPQAFKFFMEQHVENVLKSHQQRLHRKKQLENEMMRVGLSQDAQDQMRKMLCQKESNYIRLKRAKM 700
mAIS ................................................................... 536
```

FIG. 11A

BAD ORIGINAL

hLATS	DKSHLVKIKTLGIGAFGEVCLARKVDTKALYATKTLRKKDVLLRNQVAHVKAERDILAEADNEWVVRLYY	770
mLATS	....	606
hLATS	SFQDKDNLYFVMDYIPGGDMMSLLIRMGIFPESLARFYIAELTCAVESVHKMGFIHRDIKPDNILIDRDG	840
mLATS	...............................n......................................	676
hLATS	HIKLTDFGLCTGFRWTHDSKYYQSGDHPRQDSMDFSNEWGDPSSCRCGDRLKPLERRAARQHQRCLAHSL	910
mLATS	....n...................	746
hLATS	VGTPNYIAPEVLLRTGYTQLCDWWSVGVILFEMLVGQPPFLAQTPLETQMKVINWQTSLHIPPQAKLSPE	980
mLATS	..............................c..........................i............	816
hLATS	ASDLIIKLCRGPEDRLGKNGADEIKAHPFFKTIDFSSDLRQQSASYIPKITHPTDTSNFDPVDPDKLWSD	1050
mLATS	....	886
hLATS	DNEEENVNDTLNGWYKNGKHPEHAFYEFTFRRFFDDNGYPYNYPKPIEYEYINSQGSEQQSDEDDQNTGS	1120
mLATS	gs....is...s...h............h.s.	
hLATS	EIKNRDLVYV	1130
mLATS	dgn.......	966

FIG. 11B

```
hLATS   MKRSEKPEGYRQMRPKTFPASNYTVSSRQMLQEIRESLRNLSKPSDAAKAEHNMSKMSTEDPRQVRNPPK   70
mLATS2                                                                   m.at..   45

hLATS   FGHHKALQEIRNSLLPFANETNSSRSTSEVNPQMLQDLQAAGFDEDMVIQALQKTMNRSIEAAIEFISK  140
mLATS2  ..pyq...r...y........sgt.-aaa...r....e.vn.ac.qe.agr..tq.gs........y...  114

                  150       160       170       180       190       200       210
hLATS   MSYQDPRREQMAAAAARPINASMKPGNVQQSVNRKQSWKGSKESLVPQRHGPPLGESVAYHSESPNSQTD  210
mLATS2  .g.l...n..i-vrvikqtspg-.-.lastp.t.rp.fe.tg.a.-.sy.--q..-gan.--.g.aalee  175

hLATS   VGRPLSGSGISAFVQAHPSNGQRVNPPPPPQVRSVTPPPPPRGQTPPPRGTTPPPPSWEPNSQTKRYSGN  280
mLATS2  mp.qy-----------------------------------------------------ldflfpgag

hLATS   MLYVISRISPVPPGAWQEGYPPPPLNISPMNPPNQGQRGISSVPVGRQPIIMQSSSKFNFPSGRPGMQNG  350
mLATS2  agthgaqahqh..----k..---stave.sahfpgthy.rghllseqsgygv.r..s.q-nktp.dayss  251

hLATS   IGQIDEMIHQNVVPAGTVNRQPPPPYPLTAANGQSPSALQTGGSAAPSSYTNGSIPQSMMVPNRNSHNME  420
mLATS2  makaqqgppasltfpahaglytashhk-p..tppgahp.hvl.trg.-tf.ge.sa.avla.s...l.ad  319

hLATS   LYNISVPGLQINWPQSSSAPAQSSPSSGHEIPTWQPNIPVRSNSFNNPLGNRASHSANSQPSATTVTAIT  490
mLATS2  ..elq-stv--p.saapl.rrd.lqkq...-asr-.hvaf.agp-srtnsfnnpqpep.l.apn....v.  383

hLATS   PAPIQQPVKSMRVLKPELQTALAPTHPSWIPQPIQTVQPSPFPEGTASNVTVMPPVAEAPNYQGPPPPYP  560
mLATS2  a.h.lh....v...r..pQ..vg.s..a.vaa.tapate.letkegsagphpldvdyggserrc......  453

hLATS   RHLHQNPSVP---PYESISKPSKEDQPSLPKEDESEKSYENVDSGDKEKKQITTSPITVRKNKKDEERRESR 630
mLATS2  ....lpSk.eqySvdld.lCtsvqqslrggtdl.g.d..hakg.kagrd....q...vp....sr...k.... 528
```

FIG. 12A

```
hLATS   IQSYSPQAFKFFMEQHVENVLKSHQQRLHRKKQLENEMMRVGLSQDAQDQMRKMLCQKESNYIRLKRAKM  700
mLATS2  .k....y.............i.ty..kvs.rl...q..aka..ceae.e....i.y......n.......  598

hLATS   DKSMFVKIKTLGIGAFGEVCLARKVDTKALYATKTLRKKDVLLRNQVAHVXAERDILAEADNEWVVRLYY  770
mLATS2  .......................c.l..h....m.........n......................k...  668

hLATS   SFQDKDNLYFVMDYIPGGDMMSLLIRMGIFPESLARFYIAELTCAVESVHKMGFIHRDIKPDNILIDRDG  840
mLATS2  .....s.....................ev...h..........l.i....................l..  738

hLATS   IIKLTDFGLCTGFRWTHDSKYYQ-SGDHPRQDSMDFSNEWGDPSSCRCGDRLKPLERRAARQHQRCLAHSL 910
mLATS2  ...................n.....-k.n.m.....epgdl.d.v.n........t..q..qk.......... 808

hLATS   VGTPNYIAPEVLLRTGYTQLCDWWSVGVILFEMLVGQPPFLAQTPLETQMKVINWQTSLHIPPQAKLSPE  980
mLATS2  ..............k...........................p..t...l.....est....t.vr..a.  878

hLATS   ASDLIIKLCRGPLDRLGKNGADEIKAHPFFKTIDFSSDLRQQSASYIPKITHPTDTSNFDPVDPDICLWSD 1050
mLATS2  .r. .l...caadc...rd...dl......n.....r.i.k.a.p.v.t.s..m..........eesp.he 948

hLATS   DHLLLNVN-DTLNGWYKNGKHPEHAFYEFTFRRFFDDNGYPYNYPKPIEYEYINSQGSEQQSDEDDQNTGS 1120
mLATS2  asq.sakaw....as--pss....................frc...s.paesadpgdadleg ------- 1009

hLATS   EIENRDLVYV 1130
mLATS2  aaegcqp... 1119
```

FIG. 12B

BAD ORIGINAL

```
                                        LSD2a
h-LATS  MKRSEKPEGYRQMRPKTFPASNYTVSSRQMLQEIRESLRNLSKPSDAAKAEHNMSKMSTEDPRQVRNPPK-    70
LATS    ________________________________________ Mh.agekrggrpnd.yta.alesikqdltr     30

h-LATS  FGTHHKALQEIRNSLLPFANETNSSRSTSEVNPQMLQDLQAAGFDEDMVIQALQKTNNRSIEAAIEFISK   140
LATS    .evqnnhrnnq=.ytp.ryta..grndaltpdyhhakqpmepppsaspapdvv-ippppa.vgqpgag.-    97

h-LATS  MSYQDPRREQMAAAAARPINASMKPGNVQQSVNRKQSWKGSKESLVPQRHGPPLGESVAYHSE-SPNSQTD   210
LATS    i.vsgvgvgvvgv.ng.-v-p-.mtalmpnkli..p.ierdta.shyl.cs.a.dsgagssrsd..h.h-h   165
                                                 SH3-BINDING
h-LATS  VGRPLSGSGISAFVQAHPSNGQRVNPPPPPQVRSVTPPPPPRGQTPPPRGTTPPPPSWEPNSQTKRYSGN    280
LATS    thq.----s.rt.gnpgg..g-fs.s.sgfsevap.a....np.assaa.p...vpptsqayv..r.pa    229
                                                            LSD1a
h-LATS  MEYVISRISPVPPGAWQEGYPPPPLNTSPMNPPNQGQRGISSVPVGRQPIIMQSSSKFNFPSGRPGMQNG    350
LATS    lnnrppa.a.ptqrgnspvitqng.k-n.qqqlt.qlkslnly.g.gsgavvepppppyliqg.ag.aapp   298

h-LATS  TGQTDFMIHQNVVPAGTVNRQPPPPYPLTAANGQSPSALQTGGSAAPSSYTNGSIPQSMMVPNRNSHNME    420
LATS    ppppsytasmqsrqsp.qsq.s--d.rkspss.iy-..-tsa..ps.itvslppa.lakpq.rvyqarsq    364

h-LATS  LYNISVPGLQTNWPQSSSA--PAQSSPSSGHEIPTWQPNIPVRSNSFNNPLGNRASHSANSQPSATTVTAIT  490
LATS    qpi.mqsvks.qvqkpvlqtav.pq....asasnspvhvlsappsypqksaavvqqqqqaaaahqqqhqhq   436
         LSD1a        LSD1p                                     LSD2a    LSD2p
h-LATS  PAPIQQPVKSMRVLKPELQTALAPTHPSWIPQPIQTVQPSPFPEGTASNVTVMPPVAEAPNYQGPPPPYp   560
LATS    qskppt.ttppl.glnskpnc.e.psyaksm.akaatvv erdqrererdqqklangnpgrqml.....q   545

        qqqqqqqqqqqvqqqqvqqqqqqqqqqlqalrvlqaqaqr      snnnnnseikppscnnnni
```

FIG.13A

```
                                                                      LFD
h-LATS  KHLLHQNPSVPYESISKPSKEDQPSLPKEDESEKS-YENVDSGDKEKKQITTSPITVRKN—K-KDEERRESR 630
LATS    qisnsnlatt..ipvkynnnssntganssgg.ng.tgttas.stsc..ikha...pe..kis.e.e...k.f. 638

h-LATS  IQSYSPQAFKFFMEQHVENVLKSHQQRLHRKKQLENEMMRVGLSQDAQDQMRKMLCQKESNYIRLKRAKM  700
LATS    .rq............i...i..yr..ty..n...k..hk...pdgt.ie.....n..............  708

    LFD — KINASE DOMAIN
h-LATS  DKSMFVKIKTLGIGAFGEVCLARKVDT-KALYATKTLRKKDVLLRNQVAHVKAERDILAEADNEWVVRLYY  770
LATS    .........pi.v......t.vs.i..snh...m.....a...k..................n...k...  779

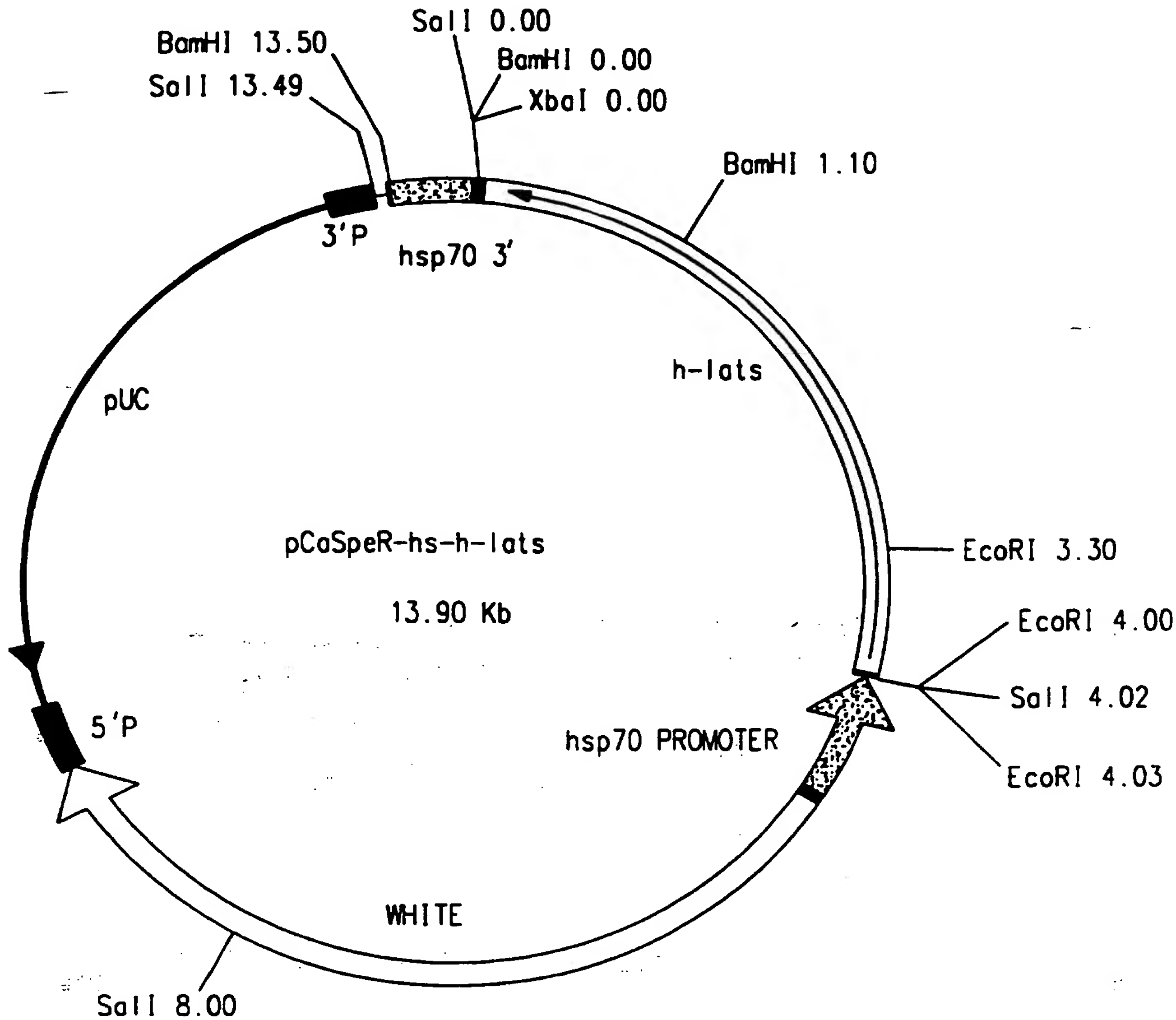
h-LATS  SFQDKDNLYFVMDYIPGGDMMSLLIRMGIFPESLARFYIAELTCAVESVHKMGFIHRDIKPDNILIDRDG  840
LATS    ...................l.....kl...e.e........v....d.......................  849

h-LATS  HIKLTDFGLCTGFRWTHDSKYYQ-SGDHPRQDSMDFSNEWGDPSSCRCGDRLKPLERRAARQHQRCLAHSL  910
LATS    .................n.....en.n.s.....e-p—.eey.e-n-.pkptv....rm.d...v.....  915

h-LATS  VGTPNYIAPEVLLRTGYTQLCKWWSVGVILFEMLVGQPPFLAQTPLETQMKVINWQTSLHIPPQAKLSPE  980
LATS    ............e.s.......y.......y...........ns.....q.....ekt.......e..r.  985

                      KINASE DOMAIN
h-LATS  ASDLIIKLCRGPEDRLGKNGADEIKAHPFFKTIDFSSDLRQQSASYIPKITHPTDTSNFDPVDPDKLWSD  1050
LATS    .t...rr..asadk....-sv..v.s.d...g...-a.m.k.k.p...e.k............e..r.n  1053
                                                          LCD1
h-LATS  DNEEENVNDTLNGWYKNGKHPEHAFYEFTFRRFFDDNGYPYNYPKPIEYEYINSQGSEQQSDEDDQNTGS  1120
LATS    .stmssgd.-vd---q.dr-tf.g.f..........kqp.dmt----------------...ap--  1096
                                  LCD2
h-LATS  EIKNRDLVYV  1130
LATS    -----....  1099
               LCD3
```

FIG.13B



SalI 0.00
BamHI 13.50
BamHI 0.00
SalI 13.49
XbaI 0.00
BamHI 1.10
3'P
hsp70 3'
h-lats
pUC
pCaSpeR-hs-h-lats
13.90 Kb
EcoRI 3.30
EcoRI 4.00
SalI 4.02
5'P
hsp70 PROMOTER
EcoRI 4.03
WHITE
SalI 8.00

FIG.14

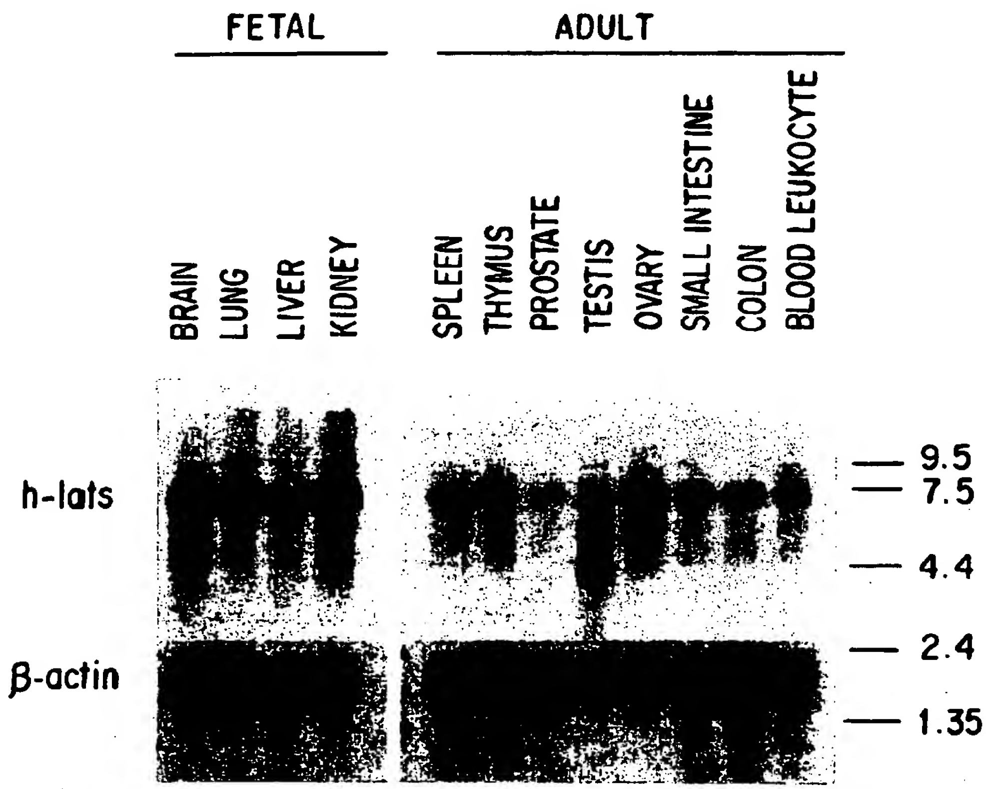

FETAL
ADULT
BRAIN
LUNG
LIVER
KIDNEY
SPLEEN
THYMUS
PROSTATE
TESTIS
OVARY
SMALL INTESTINE
COLON
BLOOD LEUKOCYTE
h-lats
β-actin
9.5
7.5
4.4
2.4
1.35

FIG.15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04101

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 11/00; C07H 21/04; C12P 21/02; C12N 5/10; A61K 38/43
US CL :530/350; 536/23.2; 435/69.1, 240.1; 514/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 536/23.2, 23.4; 435/69.1, 69.7, 240.1; 514/2; 935/9

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, IntelliGenetics
search terms: lats gene, drosophila tumor suppressor gene, sequence, nucleic acid, nucleotide, clone, expression

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	GENES AND DEVELOPMENT, Volume 9, Number 5, issued 01 March 1995, Justice et al, "The *Drosophila* Tumor Suppressor Gene *warts* Encodes a Homolog of Human Myotonic Dystrophy Kinase and is Required for the Control of Cell Shape and Proliferation", pages 534-546, see entire document.	1, 3, 6-8, 10-19, 23-25, 28-32, 34-36, 38-39 ------------- 2, 4-5, 9, 26-27, 33, 37, 40-52, 78
X	EMBO JOURNAL, Volume 11, Number 6, issued June 1992, Yarden et al, "*cot-1*, a Gene Required for Hyphal Elongation in *Neurospora crassa*, Encodes a Protein Kinase", pages 2159-2166, see entire document.	7-8, 10-11, 14-19, 28-30, 32, 35, 39

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

09 JULY 1996

Date of mailing of the international search report

25 JUL 1996

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Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 266, Number 19, issued 05 July 1991, Shortridge et al, "A *Drosophila* Phospholipase C Gene that is Expressed in the Central Nervous System", pages 12474-12480, see entire document.	7-8, 10-11, 14-15, 17-19, 28-30, 32, 35, 39
X	GENE, Volume 104, Number 1, issued 1991, Toyn et al, "The Cell-Cycle-Regulated Budding Yeast Gene *DBF2*, Encoding a Putative Protein Kinase, has a Homologue that is Not Under Cell-Cycle Control", pages 63-70, see entire document.	7-8, 10-11, 14-15, 17-19, 28-30, 32, 35, 39
X, P ---- Y, P	DEVELOPMENT, Volume 121, Number 4, issued April 1995, Xu et al, "Identifying Tumor Suppressors in Genetic Mosaics: the *Drosophila lats* Gene Encodes a Putative Protein Kinase", pages 1053-1063, see entire document.	1, 3, 6-8, 10-19, 23-25, 28-32, 34-36, 38-39 ---------- 2, 4-5, 9, 26-27, 33, 37, 40-52, 78

INTERNATIONAL SEARCH REPORT

Inten   ›nal application No.
PCT/US96/04101

Box I Observations where certain claims were found unsearchable (C ntinuation f item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-19, 23-52, and 78

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

unity of invention is lacking.

Groups V and XIV contain claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention for the above reasons, which explain why the compositions used lack unity and are not so linked as to form a single inventive concept under PCT Rule 13.1. If the fee for searching Groups V or XIV is paid, the first named embodiment,the anti-lats antibody, will be searched. In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species for claims 66-67, 69, 100-103 are as follows:
A) anti-lats antibody.
B) lats derivative or analog.
C) lats antisense nucleic acid.
D) a nucleic acid comprising a portion of the lats gene.

In Group V, the following claims are generic: claims 66-67, 69.
In Group XIV, the following claims are generic: claims 100-103.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04101

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-19, 23-52 and 78, drawn to a purified lats protein, derivative, analog, or fragment, a chimeric protein, an isolated nucleic acid, a recombinant cell, a method of producing the lats protein and a pharmaceutical composition and a kit that comprises a lats protein.

Group II, claims 20-22, 56-57 and 77, drawn to an antibody, a molecule comprising antibody fragments and a pharmaceutical composition and a kit comprising these antibodies/fragments.

Group III, claims 53-55, 70-71 and 77, drawn to pharmaceutical compositions comprising a therapeutic nucleic acid, an oligonucleotide, a recombinant cell and a kit comprising the nucleic acid probes/primers.

Group IV, claims 58-65, drawn to a method of treating a disease state by administrating a molecule that promotes lats function.

Group V, claims 66-69, drawn to a method of treating a disease state by administrating a molecule that inhibits lats function.

Group VI, claim 72, drawn to a method of inhibiting expression of a nucleic acid with an oligonucleotide.

Group VII, claims 73-76, drawn to a method of diagnosis of a disease by screening aberrant levels of lats RNA or protein using nucleic acids or proteins or antibodies.

Group VIII, claims 79-80, drawn to a method to increase cell growth in plants.

Group IX, claims 79 and 81, drawn to a method to increase cell growth in animals.

Group X, claim 82, drawn to a method of screening for lats ligands.

Group XI, claims 83-85, drawn to transgenic plants.

Group XII, claims 83, 85, 92-95 and 99, drawn to transgenic animals and method of making.

Group XIII, claims 86-91 and 96-98, drawn to a method of identifying a tumor suppressor gene.

Group XIV, claims 100-103, drawn to a method of inhibiting cellular senescence in a subject.

Claim 77 has been placed in both Groups II and III. The antibody embodiment will be searched with Group II. The nucleic acid embodiment will be searched with Group III.

Claim 79 has been placed in both Groups VIII and IX. The plant embodiment will be searched with Group VIII. The animal embodiment will be searched with Group IX.

Claims 83 and 85 have been placed in both Groups XI and XII. The plant embodiment will be searched with Group XI. The animal embodiment will be searched with Group XII.

The inventions listed as Groups I-XIV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I is directed to purified lats protein, analogs, fragments, chimeric constructs, to the DNA that encode them and to a pharmaceutical composition and kit, which is the first appearing product, method of making and method of using. The special technical feature is the disclosed protein and DNA sequences. Group(s) II-III, XI-XII are drawn to structurally different products which do not share the same or a corresponding technical feature. Group(s) IV-X and XIII-XIV are drawn to methods having different goals, method steps and starting materials, which do not share the same or a corresponding special technical feature. Note that PCT Rule 13 does not provide for multiple products or methods within a single application. Since the special technical feature of the Group I invention is not present in the Group II-XIV claims, and the special technical features of the Group II-XIV inventions are not present in the Group I claims.